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***In Vitro* Isolation and Propagation of Mammatoxin-Resistant Aspen**

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IN VITRO ISOLATION AND PROPAGATION OF MAMMATOXIN-RESISTANT ASPEN

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ABSTRACT

Hypoxylon canker (*Hypoxylon mammatum* Wahl. Miller) is considered to be the most serious disease of quaking aspen (*Populus tremuloides* Michx.). *H. mammatum* has been shown previously to produce a host-selective toxin that putatively has been identified as a determinant in the disease. This has suggested that "mammatoxin" might be substituted for the pathogen in order to screen varieties of aspen for disease resistance.

A method is described in which mammatoxin was used *in vitro* to screen tissue cultures of aspen for resistance to the necrotic effects of the toxin. By a novel organogenesis procedure, plants were regenerated and established in soil from surviving cultures. After 6-32 weeks of growth, challenge of these propagules with mammatoxin in a leaf puncture bioassay revealed that the resistance expressed *in vitro* was maintained in the plant.

The origin of the mammatoxin resistance was demonstrated to reside in the seedling populations cultured. By establishing shoot cultures from a single cotyledon and growing the remainder of the seedling, the mammatoxin response of the propagules could be compared to that of the donor plant. When cultured on toxin-free medium, 16% of the ortet-ramet families exhibited significant intraclonal variation in the mammatoxin response. However, all propagules regenerated from toxin-resistant cultures were resistant to mammatoxin, and corresponded to toxin-resistant seedlings. The tissue culture screening system allows for convenient roguing of seedlots and subsequent propagation of individuals expressing mammatoxin resistance.

INTRODUCTION

OBJECTIVE

Members of the genus Populus represent some of the fastest-growing trees in temperate climates throughout the world. When the heavy dependence on Populus of Korea, China, India, Pakistan, and Europe is considered, it becomes apparent that more people in the world depend on Populus species as a source of fuel, fiber, and solid wood products than any other genus of forest trees.

While many Populus species have the capacity for outstanding volume growth, their potential is often not realized due to diseases. Ironically, it is probably the rapidity of growth that tends to produce a succulent, and therefore appealing, host for a depressingly long list of pests. If one eliminates the 50 or so diseases associated with old age (beyond rotation), there are still approximately 80 diseases that have been cataloged on Populus species in the United States alone[1].

In commercially important forest species, where exogenous control of pests is generally not cost-effective, breeding for resistance becomes the only viable management option. In this regard, it is fortunate that poplars enjoy outstanding opportunities in breeding for disease resistance. These opportunities arise not only from the variability within a particular species, but also from the ease of interspecific hybridization, enabling infusion of disease resistance from one species into another.

However, in sharp contrast to agronomic crops, progress in breeding for disease resistance (or any trait of proven value) in trees is hampered by their immensity and longevity.

The problem associated with the long life cycle of trees is twofold. The first part of the problem is that trees usually take many years to reach sexual maturity. Because of this long generation time, selection and its subsequent utilization must wait until promising individuals at least reach sexual maturity. This problem becomes particularly acute when it is appreciated that disease resistance alone is of little value to the pulp and paper industry unless combined with other desirable characteristics such as specific gravity, growth rate, and form. These characteristics tend to be quantitatively inherited, (i.e., polygenetic) and can require many generations to achieve satisfactory combinations.

The other aspect of the problem of longevity is that trees must remain in place for many years before harvest. During that time, they are continually confronted with pests and environmental conditions that may predispose them to infection. Furthermore, these pests are capable of turning over many generations in the life span of a tree, enabling them to test a myriad of genetic recombinations against the fixed genome of the tree.

The immensity of trees directly hampers tree breeding by making intense selection difficult. In agronomic crops, thousands of individuals can be examined on a single acre. Trees on the other hand require acreage that is at least an order of magnitude larger for a comparable number of individuals[2]. Furthermore, if large acreages required for stringent selection are employed, environmental factors such as site and microclimate variability may confound the phenotypic expression of the desired genotype.

It is precisely these problems that make <u>in vitro</u> selection an attractive approach in screening for disease resistance. By use of a tissue culture system,

large organisms can be reduced to a cellular level, enabling the examination of many genomes in negligible space. The use of an *in vitro* system further implies strict and reproducible environmental control as well as asepsis which can enable the establishment of pathogen-host cultures free from other interfering organisms.

Perhaps the most intriguing (and controversial) aspect of *in vitro* screening for desired traits that has recently emerged is that the tissue culture system, acting alone and without the application of mutagenic agents, can provide a rich source of variation. Thus, plants derived from the *in vitro* culture of somatic tissue do not always exhibit characteristics that are true-to-type. The acceptance of this so-called somaclonal variation[3] has led to the notion that *in vitro* screening selectively permits proliferation of a somatic cell-derived variant expressing a desired trait that was absent or otherwise masked in the original genome.

The potential benefits of *in vitro* techniques have not been realized in forest species owing to the difficulty in regenerating plants from tissue and cell cultures of many of these species. One notable exception to this is *Populus*. Plants have been regenerated in many species from tissue[4-7] and cell[8-14] cultures representing both the sporophytic and gametophytic (anthers) generations. Undoubtedly, success came quickly in tissue culture of *Populus* species owing to its propensity for vegetative propagation. This success has led to the commercialization of *Populus* tissue culture[7,15] - a development that is not presently viable with any other pulpwood species. In fact, the annual *in vitro* production of one triploid hybrid aspen clone (*P. tremula* x *P. tremuloides*) Astria (50,000)[7] is comparable to the sum of all coniferous tissue culture propagules produced globally up to and including 1983 (70,000)[16].

The increasing importance of *Populus* species in the pulp and paper industry underscores the need for development of disease resistant varieties. The success of *in vitro* culture of *Populus* and the tantalizing results obtained to date with *in vitro* screening for disease resistance in agronomic crops beg the application of this technique to *Populus*. Broadly stated, the objective of this work was to demonstrate that an *in vitro* screening technique could be used to identify tissue cultures of *P. tremuloides* exhibiting tolerance to a stress agent, and subsequently regenerate plants that retain this resistance. In an abstract sense, the stress agent could be physical, chemical, or biological; here, a phytotoxin (mammatoxin) was chosen.

As measured by a biological assay, an *in vitro* screening procedure was developed and utilized to produce mammatoxin-resistant aspen. However, the extent to which the observable reaction to mammatoxin is causally related to the incidence of the hypoxylon canker is beyond the scope of this work. Although evidence indicting mammatoxin as a determinant in this disease is provided, it should be noted that mammatoxin has not been demonstrated conclusively to be a determinant in the disease.

The choice of tissue for explanting (seedlings), and the increasingly pervasive phenomena of somaclonal variation requires an investigation into the origin of the resistance response. "Origin" in this sense does not mean the genetic or molecular basis of the resistance response, but rather if the trait was present before *in vitro* manipulation or was produced by the tissue culture system. Aspen seedlings, even from full-sib crosses, can exhibit substantial phenotypic (and presumably genetic) variation. Thus, each seedling can be considered a clone, and an adequate assessment of the resistance response of the seedlings employed must be made.

From a practical standpoint, a micropropagation method for producing plants from seedling explants must first be developed before any *in vitro* screening work can be performed. Seedling explants in *Populus* have been ignored until now, owing to the ease of obtaining or regenerating suitable material from older trees. Given the combined morphogenic potential of juvenile tissue and *Populus*, a micropropagation scheme was readily devised. In the method developed here, the two criteria that had to be met were reliability and a reasonable degree of productivity. The criterion of productivity is necessary to ensure the recovery of plants, should toxin-induced stress cause the morphogenic capacity to deteriorate. It should be noted here that productivity is not equated with the optimization of this process for clonal mass production of plants.

Finally, a comparison of the response to mammatoxin exhibited by seedlings *versus* their tissue culture derived counterparts affords an opportunity to passively examine a basic tenet of tissue culture of woody species - namely, are plants derived from tissue cultures true-to-type? This has always been an assumption in micropropagation procedures based on adventitious bud formation in woody species, but data that addresses this assumption are sorely lacking. To my knowledge, this represents the first time that a phenotypic response of a tissue culture propagule is directly compared to the seedling from which is was derived.

BACKGROUND

The Hypoxylon Canker Problem

Hypoxylon canker [*Hypoxylon mammatum* Wahl. Miller syn. *H. pruinatum* (Klotzsch) cke.] is generally regarded as the most serious disease of quaking aspen (*P. tremuloides* Michx.). Considering that quaking aspen has the widest

geographic range of any forest species in North America[17], and that hypoxylon canker occurs on aspen throughout its entire range (except for the interior of Alaska[18]), Hypoxylon mammatum may well be the most prevalent, aggressive pathogen of a woody species on this continent. Although hypoxylon canker is widespread, damage caused by the disease ranges from slight in the western and southwestern United States[19] to acute in the Lake States[20,21] and the northeastern United States[22]. The disease causes mortality by girdling the bole - killing it outright or predisposing it to wind breakage. Anderson[20], in summary of a U.S. Forest Service study initiated in the Lake States region in 1949, estimated that hypoxylon canker killed 1-2% of the standing volume each year. In 1964, this loss in volume represented 31% of the annual growth and was a greater volume than that utilized by the pulp and paper industry. In the most recent Lake States survey, Marty[21] estimated a slightly higher annual mortality of 3% and also reported that 12.1% of all trees are currently infected. On an annual basis, this corresponded to 300 million cubic feet, an amount approximately equivalent to the annual growth. When this is compared to the 280 million cubic feet utilized by the pulp and paper industry in 1983[23] it can be readily appreciated that the amount of aspen available for utilization could be doubled if hypoxylon canker were eradicated. Although it is difficult to affix a monetary value to wood lost that otherwise might not have been utilized, at current stumpage prices ($0.05/ft^3;[24]) it would represent an amount of wood valued at 15 million dollars annually.

The difficulty associated with an economic estimate of wood loss arises because the Lake States are currently enjoying a surplus of aspen. In fact this "glut" has led to the opportunistic appearance of waferboard manufacturing facilities that utilize a 100% aspen furnish. In 1981, these plants manufactured 520

million square feet of board (3/4-inch basis), equivalent to approximately one million cords of aspen[25]. When this is coupled with the 2% annual increase in pulp production[25] predicted for this region, it seems likely that demand for aspen will continue to be strong.

While many people are of the opinion that the supply of aspen will be adequate to meet this demand, it has been noted that much of this resource is overmature and as such the current glut could turn into a shortage over a relatively short time interval (1995-2005)[26]. Thus, while the Lake States presently can afford the luxury of the tremendous loss of aspen caused by hypoxylon canker, the future of the Lake States aspen resource may soon demand hypoxylon canker resistant varieties.

The Etiology of Hypoxylon Canker

In testimony to the magnitude of the hypoxylon canker problem, numerous reports on the host range[27-31], mode of infection[28-29,32-36], and histology of pathogenesis[32,37-39] have appeared over the last several decades. Hypoxylon canker shares a commonality with other diseases in that the relationship between the genetics of the host, genetics of the pathogen, and the environment are inextricable from each other.

Variation in host susceptibility was reported by Copony and Barnes[40], who found substantial differences in the natural canker incidence among *P. tremuloides* clones growing on a variety of sites. Examination of half-sub progeny groups by Valentine[41] suggested that heritability for several resistance mechanisms was low - perhaps artificially so due to modulation caused by variability in the pathogen. Curiously, while *P. tremuloides* is the principal host of *Hypoxylon mammatum*, intra- and intersectional crosses with *P. tremuloides* generally result

in enhanced susceptibility to the disease in the hybrids relative to the pure species being crossed with quaking aspen[28]. This has suggested that susceptibility to hypoxylon canker is dominant, but simple dominance was not demonstrated in progeny groups of P. tremuloides, P. grandidentata, and both reciprocal, intraspecific hybrids[42].

In this work, one of the deciding factors in the choice of seedlings as explant material was based on observed variation in natural canker incidence among full-sib progeny groups of quaking aspen over a 25-year period[31]. Specifically, some parent trees had been identified that consistently produced progeny that were susceptible or resistant across a wide range of crosses.

Variation in the pathogenicity and virulence of the fungus has been investigated through use of mass isolates taken from infected host tissues in different locales or from single ascospore cultures derived from these isolates. As a member of the family Ascomycetes, Hypoxylon mammatum reproduces sexually by production of ascospores that are discharged from perithecial stromata. These haploid ascospores arise from mitotic division of the tetrads produced by meiosis, and are contained in packages of eight, termed asci. Each of these eight spores can be dissected from the ascus and segregated prior to discharge, and cultures can be derived from single spores. Cultures derived in this way have been shown to have profoundly different cultural morphologies and nutritional requirements[43], as well as pathogenicity[43-46]. While avirulence was associated in some cases with a conidial-type morphology in vitro, substantial variation in virulence, usually measured as canker length after artificial inoculation, was observed in cultures exhibiting a similar mycelial-type morphology[44].

In addition to the above, isolates taken from cankers on one species (P. grandidentata) produced cankers that elongated faster when inoculated on P. tremuloides[42].

Superimposed on the genotypic variability of the host-pathogen interaction is a strong environmental component. In many instances this has obscured definitive statements on the etiology of the disease, and has led to a large collection of seemingly unrelated observations on the disease. Many of these observations (e.g., effect of stand density, canker location, sudden outbreaks of disease, effect of moisture stress) can be unified under the concept that Hypoxylon mammatum is a wound parasite[32,37], infecting the sapwood and invading the bark from within[47,48]. The prerequisite of wounds and the observation that only dead bark is invaded appears to arise from the observation that the underlying green layer of bark (phelloderm) contains potent fungistatic activity. Hubbes[49,50] identified the fungistatic activity with pyrocatechol and two unknown glycosides which yielded benzoic and salicylic acid upon hydrolysis. The concentration of these compounds was highest in bark near the base of the tree, and lowest in the bark associated with young branches near the crown.

The general inhibition of spore germination by these compounds[37] or aspen bark meal[51] has been taken as evidence that exposed xylem is necessary for infection. Ascospores have been successfully germinated in sapwood of aspen[33,52], and on bark meal derived from young branches[52].

The hypothesis that the underlying green layer of bark acts as a protective sheath around the tree can be used to account for some of these seemingly unrelated observations. In general, any environmental agent that leads to

discontinuity of the phelloderm, or alternatively disrupts the normal wound response of the host can potentially contribute to conditions leading to successful infection. Canker location near the crowns of trees or on young branches can be understood by the reduction in fungicidal activity in the bark of younger material. It is also these same locations that are a favorite location for the egg-laying activities of at least two species of insects, Saperda inornata and Magicicada septendicim. High incidence of hypoxylon canker has been reported in galleries of both of these insects[35,53]. M. Ostry is of the opinion that these oviposition wounds provide an ideal microchamber for spore germination, protected from adverse conditions and beneath the inhibiting bark layer[54]. The fluctuations in populations of these insects may also account for periodic outbreaks of hypoxylon canker as well as the observation that canker incidence is inversely proportional to stand density[55], as these insects are "sun-loving" and prefer open areas[56]. Cankers once established on these branches, have been shown to have the ability to travel down to the bole and girdle the tree[35].

Another long standing observation on hypoxylon canker is that cankers are often located at branch axils, frequently with the stub of the dead branch in the middle of the canker. French and Oshima[57] have suggested that the areas near branches have discontinuities in the layer of bark containing the inhibiting substances and thus might be avenues for successful entry into the xylem. Graham has noted that branch axils are also sites for activity by a number of boring insects[58].

The location of the "typical" canker at the branch axils highlights a problem associated with investigating disease of organisms with long life cycles.

When confronted in the field with a canker at a branch axil there is often no clue as to how the canker came to be situated at this site (e.g., was infection initiated on the branch, and subsequently migrated to the bole or, alternatively, did infection originate at the axil?). Knowledge of canker origination may provide information on the relative importance of different infection schemes, but in situations like the above, the researchers are often left guessing at the importance of various infection mechanisms.

A final observation that may be best understood in terms of hypoxylon canker as a wound parasite is the effect of moisture stress. Canker incidence has not been correlated with site index[20,21,59] but has been found to be negatively correlated with soil moisture[60]. Bier[61] has experimentally demonstrated canker growth inhibition by maintaining high bark turgidities, and he suggested that during periods of moisture stress, the wound healing processes of the host may be hampered, which may contribute to the inability of nonpathogenic microflora to successfully compete with H. mammatum[62].

From the above discussion an appreciation can be gained of the confounding of the genetics of the host-parasite relationship by the environment. In effect, any imaginable environmental process that creates wounds or inhibits repair can contribute to the potential for successful infection. A particularly unique example of this was an outbreak of hypoxylon canker in P. tremuloides that occurred after a hailstorm in Minnesota[63].

In addition to the complexities of host, pathogen, and environmental interaction, attempts to obtain information on the etiology of the disease through garden plot or cut-branch studies have been restricted to artificial inoculations with mycelia. This has been done because of the previously

mentioned difficulty in achieving ascospore germination in aspen bark or wood, in contrast with the relative ease of infection employing mycelia. However, ascospores have been assumed to be the principal vector in the spread of the disease, owing to their ubiquitous presence on the bark of P. tremuloides[32,58]. Mycelial vectors have been tenuously identified as insects or even woodpeckers[59] but this has not been pursued extensively. Thus, after 40 years of intensive study, even the most fundamental characteristics of the infection process are still unresolved.

The Possible Role of Mammatoxin as a Determinant in the Disease

In 1964, Hubbes reported that a diffusable substance produced by H. mammatum prevented callus formation and hastened the blackening in wounded bark tissue of quaking aspen[37]. Later, Bagga and Smalley[34] demonstrated that this substance was water soluble, heat-stable, and capable of not only producing symptoms characteristic of the disease, but would enable establishment of infection by ascospores when exogenously added to spore inoculum. This latter observation was remarkable given the difficulties in establishing infection via ascospores. Additional confirmation by Schipper[64] of the ability of this "mammatoxin" to elicit symptoms of the disease in the absence of the pathogen and, only on species susceptible to hypoxylon canker, led to the belief that mammatoxin was necessary for successful infection.

Diseases that have host-selective toxins as determinants are unique to plants and no analogy exists with animal diseases[65]. In general, they are compounds of low molecular weight (500-2000) with their toxic effects restricted to hosts susceptible to the disease. Diseases with host-selective toxins have received a great deal of attention because many diseases of economically important crops fall into this category and, toxins can simplify studies on

pathogenicity by substituting for the pathogens. As a result, innumerable reviews and several books devoted to the subject are available[66,67].

The mechanisms by which pathotoxins act as determinants in plant diseases are largely unknown, but extensive work with toxins from Helminthosporium victorae and H. maydis (race T) has suggested an interference with energy generation systems in the chloroplast and mitochondria, respectively[68]. Although these organelles appear to be the targets, the toxins appear to initiate a chain of events and the difficulty arises in determining the penultimate event.

A large part of the problem with determining how toxins participate in the infection process rests with the fact that a toxin is usually not a single molecule but a collection of closely related molecules each with complex structures. The structures of only a few of these molecules have been established and therefore knowledge of toxin titres has to be inferred, usually through biological assays that can often be influenced by factors not associated with the disease or the toxin. Even when purification techniques and concentrations are exactly known, arguments have been forwarded that the pathogen does not purify these molecules before applying them to the host, thus toxic artifacts may be created in the purification process.

Due to the above considerations, little more than implication of the causal role of toxins has been made by taking advantage of their extreme selectivity. Thus, effects of toxins on closely related, but nonhost species can be examined for differences by comparison to the host under similar experimental conditions.

Perhaps the strongest evidence indicting toxins as causal agents comes from the ease of obtaining mutant pathogens lacking only the ability to produce

toxins. An outstanding example of this was a mutant bacterium of Pseudomonas phaseolica lacking the ability to produce a chlorosis inducing toxin that was isolated and subsequently shown to be unable to invade the host systemically as did the wild type[69].

Evidence that hypoxylon canker has a toxin that is a determinant also suffers the same problems noted above. Thus, evidence is indirect and circumstantial. To a large extent, the deterministic role of mammatoxin is implied by analogy to other diseases in which host-selective toxins have been substantiated as a determinant. Table 1 is an attempt to summarize the literature on the implication of mammatoxin as a determinant in the disease. The evidence provided suggests, by analogy, that hypoxylon canker exhibits the characteristics of other diseases that have toxins as determinants. In this context, determinant is not differentiated into a primary (effecting pathogenesis) or a secondary (effecting virulence) role.

Entry 1 in Table 1 is so fundamental it hardly needs stating. Clearly, mammatoxin could not be a determinant if it were not present in active cankers. The presence of mammatoxin in cankers and in vitro cultures of H. mammatum suggests the toxin is a regular metabolite, and not produced in response to host. This is also the case with other toxin-determinant diseases.

Entry 2 addresses a feature which many consider a "tip-off" that toxins are causally involved in disease development. Many diseases in which toxins are thought to be determinants have been given the graphic monickers "blight," "rot," or "wilt" related to the appearance of infected plants. Often, discoloration or other symptoms appear in areas considerably removed from the infection site. Furthermore, cell-free extracts of cultures of the pathogen will also produce these characteristic symptoms.

Table 1. Implication of the determinant role of mammatoxin in the development of hypoxylon canker.

Entry	Observation	Reference
1	Mammatoxin isolated from cankers	64
2	Mammatoxin elicits symptoms of the disease in the absence of H. mammatum	34,37,64,73
3	Symptoms elicited only in species susceptible to hypoxylon canker	28,64
4	Infection by ascospores achieved by exogenous addition of mammatoxin	34
5	Avirulent isolates made virulent by exogenous applications of mammatoxin (and vice versa)	70
6	Isolates that produce the most toxin were the most virulent	71
7	Mammatoxin active at physiological levels only on P. tremuloides	72
8	Dormant stems resist infection and also fail to develop necrosis in response to mammatoxin	73
9	Natural incidence of hypoxylon canker correlated with leaf puncture bioassay	74,75

Cell-free extracts of H. mammatum have been shown on several occasions to mimic events similar to infections established artifically and naturally. As noted previously, these are the inhibition of callus formation associated with a wound and the ability to hasten discoloration in wounded bark tissues. As early as 1945, Gruenhagen observed in vivo that a blackening reaction preceded the growth of the mycelia fan[32].

The host selectivity of the necrotic response (Entry 3) to mammatoxin is also analogous to the behavior of toxin-determinant diseases. Perhaps the most infamous example of selectivity is the case of southern leaf blight in corn

caused by Helminthosporium maydis (race T) that decimated the crop in 1971 in the United States. In corn hybrids, the simple replacement of Texas male sterile by normal cytoplasm resulted in resistance to the blight. The necrotic effect of mammatoxin has likewise been demonstrated to be most severe in P. tremuloides. However, other Leuce poplars that are erstwhile hosts, and interspecific hybrids with P. tremuloides parentage, also respond necrotically, but only to a limited degree[64,73]. It is of interest to note that these examinations of the response to mammatoxin were performed by treating leaves (not normally a site of infection) with the toxin. The observation that the response exhibited by leaves to the toxin was similar to that exhibited by stems is consistent with the hypothesis that toxins act on fundamental, cellular "housekeeping" systems. Hence, it is not unreasonable to expect that similar toxin reactions would be exhibited irrespective of cell type. This feature is potentially critical to the in vitro screening of cell and tissue cultures for disease resistance. If infection depends on the presence of a specific organ or cell type not present in vitro, the likelihood of success may be diminished.

Entries 4 and 5 in Table 1 are attempts to directly indict the role of toxin in the infection process. Entry 4, while intriguing from the previously mentioned standpoint of mammatoxin ensuring successful infection with ascospores, is highly circumstantial. Wheeler has pointed out that exogenous additions of toxin preparations to spores may enhance germination of spores or otherwise yield a more aggressive pathogen by means unrelated to the role of the toxin in the disease[76].

Entry 5 is likely to be more relevant in the infection process as it examines the role of toxin on the fungal morphology that is more prevalent in the disease. In this regard, the growth of the mycelia is the means by which

the fungus spreads throughout the tree. Conidia[34] and ascospores[64] have been previously shown to be nearly devoid of toxin, a product of the mycelia[64]. As noted, ascospores seem to be the most likely vector for transmission of the disease, but it is only after germination and mycelia production that infection is established. Conidia, although produced in active cankers, are generally associated with advanced stages of infection.

Examination of the role of the toxin in mycelial growth eliminates the potentially artifactual effects of mammatoxin on germination of vegetatively and sexually derived spores. Intuitively, it seems more reasonable to examine the role of mammatoxin in mycelia in which they are produced.

Schipper took advantage of the fact that mammatoxin is readily excreted by mycelia when grown in liquid cultures. Removal of the mycelia, followed by maceration and extraction, revealed no toxin activity remaining in the mycelia[70]. In this way, infection by artificial inoculation could be compared with a pathogenic isolate both lacking and containing toxin, but which otherwise were identical. Toxin-free mycelia were found to be unable to infect, but pathogenicity could be restored by exogenous addition of toxin. These experiments may be the closest attempt at mimicking the behavior of mutants lacking only the ability to produce mammatoxin.

The possibility that mammatoxin contributes to virulence rather than pathogenicity is revealed in Entry 6. Virulence, as measured by canker length, was found to be highest in isolates producing the greatest amount of toxin. That pathogenicity was not correlated with toxin production may be a consequence of the inoculation procedure employed. Artificial inoculation was performed by boring a small hole (4-6 mm diameter) in bark and sealing the inocula in the

wound to prevent desiccation. Although a hole this size may seem small, it is a crater when compared to an oviposition site, and may be substantially more difficult for the tree to close efficiently and quickly by callus formation. Although artificial inoculations have and will continue to contribute to understanding hypoxylon canker, this procedure may live up to its name in terms of evaluation of pathogenicity.

Entry 7 suggests mammatoxin is analogous to other host-selective toxins due to an ability to elicit necrosis at physiological levels on the order of nanograms. Seedlings of oat cultivars susceptible to Helminthosporium victorae have been shown to be sensitive to victorin at levels on the order of 1 ng/mL, whereas resistant cultivars will tolerate 400,000 times higher concentrations without apparent damage[77]. Stermer[72] has shown mammatoxin to be active in preparations containing 10 ng dry weight of crude extract, and observed that one clone could tolerate 500 times this level without changes in respiration rates or electrolyte leakage in toxin treated cells.

Entries 8 and 9 are not related to the role of the toxin in disease development per se, but rather are attempts to relate phenomenological features of hypoxylon canker to the necrotic response of aspen tissues to mammatoxin.

The ability of dormant stems to resist infection is still an open question. Several independent investigators have been unable to induce cankers by artificial inoculation (with mycelia) of stems in the winter[28] and Hubbes found this was precisely when pyrocatechol concentrations were the highest[49]. On the other hand, Bier argued that during the winter, bark turgidity is lowest and should be an opportune time for infection because the ability of the host to callus is lowest[62]. On the other hand, ascospores are ejected from stromata year round,

especially after rain or snow[78]. Stermer has shown that the necrotic effects of mammatoxin are not elicited in dormant stems. This suggests that mammatoxin is involved in pathogenicity, as cankers have the ability to grow, albeit at a slower rate, during the winter[46].

That the natural incidence of hypoxylon on stems is positively correlated with the necrotic effect of mammatoxin on leaves is one of the fundamental assumptions of this work (Entry 9). This implies that a quick, simple, and convenient bioassay performed with culture filtrates of H. mammatum can be used in place of the organism, and at the same time, eliminate the years required to evaluate natural canker incidence. While establishment of the link between toxin response and disease resistance was not an objective in this work, this observation certainly provided an impetus for in vitro screening.

Bruck and Manion[74] found that the response to mammatoxin (as measured by lesion diameter in a leaf puncture bioassay) decreased as the natural field canker incidence decreased. The magnitude of this decrease in lesion diameter was rather small, averaging about 5 mm for 70% natural canker incidence to 3.5 mm for 0% canker incidence. While the authors did not state the number of years of observation that went into the determination of natural incidence, it is interesting to note that their highly resistant clone still responded necrotically to mammatoxin. This suggests that other factors may be involved in hypoxylon canker development that are not reflected in the bioassay. However, in a separate study[75] comparing the natural canker incidence in five clones with canker length, branch death, and callus formation (all resulting from artificial inoculation), in addition to the leaf puncture bioassay, the most significant correlation to natural infection was the bioassay.

The observations presented in Table 1 seem sufficient to warrant attempts at selection for toxin-resistance. Whether mammatoxin is involved in pathogenicity or virulence may be more a question of semantics. Given the assumption that ascospores are the means by which the disease is spread, and spores are virtually devoid of mammatoxin, it would seem that unknown, initial conditions for spore germination must be met prior to any toxin mediated events. Once hyphae are produced and toxin is synthesized several possible roles of toxin can be postulated.

Probably the most attractive role was first postulated coincident with the discovery of the toxin - namely, mammatoxin inhibits or disrupts the normal wound response of the host. The necessity of wounds for establishment of infection, and the observation *in vivo* that callus formation is a resistance mechanism[41,46], testify to the importance of this on an organismic level. Callus formation as a resistance mechanism has been shown to be qualitatively inherited (i.e., mono- or oligogenetic) and it is pertinent to note that phytotoxin resistance in other genera is also under the control of single or small groups of genes[65]. That *P. tremuloides* is the principal host of *H. mammatum* might be a consequence of the observation that quaking aspen is unique in Leuce poplars in its relative inability to proliferate copious amounts of callus in response to wounds[28].

Of the other resistance mechanisms that have been investigated *in vivo* (early branch death, retardation of canker elongation, and reduced sporulation of established cankers[41,46,79]), early branch death and retardation of canker elongation implies that toxin has a role in virulence. Early branch death implies extreme sensitivity to the necrotic effects of the toxin, such that a canker, if established on a branch, may never migrate to the bole. It should be

noted that this is a conditional resistance mechanism, dependent on the importance of branches as sites for initial infections. Branches have been indicated as primary infection sites from studies on the role of insect wounds[35] and the effect of branch pruning on canker incidence[80]. In addition, it may be significant that P. grandidentata, a species with a propensity for natural pruning[81], generally has a lower incidence of hypoxylon canker despite its sensitivity to mammatoxin[64]. Early branch death and canker length have been positively correlated and are both indicative of virulence[46], so it is canker location that determines if this is a sign of resistance or susceptibility. Recall from the earlier discussion on the "typical" canker location (branch axil) that it is unclear where natural infection initiates.

In contrast, retardation of canker elongation appears to be associated with a tolerance to any virulent effects of the toxin. Reduced formation of perithecial stromata was also associated with a slower rate of canker development, a factor which may be important to the secondary spread of infection[79]. "Practical resistance" by these mechanisms might be as valuable as immunity to hypoxylon canker if they allow a tolerable loss of wood volume over the rotation age.

In light of the above, the role of mammatoxin can be implicated in all of the resistance mechanisms. By analogy to other toxin-determinant diseases, only inhibition of callus formation, by virtue of its qualitative nature of inheritance, seems the most likely for the primary effect of the toxin, suggesting its importance in pathogenicity. Excluded from the above discussion were the uninvestigated mechanisms of insect resistance[35] and the possibility that mammatoxin may somehow act to reduce the fungicidal activity of the bark. Specific resistance to insects is outside the realm of the host-pathogen interaction except for the potential importance of reducing wound occurrence. Given the

wide variety of insect genera that plague quaking aspen[58], it seems doubtful that a broad ranging resistance to all these pests could be bred and selected. The possibility that high levels of catechols or phenolics impart resistance also appears unlikely because the fungus invades the bark from within, presumably after the cambium has been damaged to the extent that the bark is in a state of decline. In short, the utility of *in vitro* selection is probably the ability to select for a resistance mechanism based on cellular function. As noted previously, host-selective toxins are generally active at the cellular level and not dependent on tissue or organ levels of development. It does not seem unreasonable to assume then that selection on a cellular level might encompass other resistance mechanisms dependent only on morphology or certain tissue. It is noteworthy here to consider the case of *P. deltoides*. Eastern cottonwood is not considered a host of *H. mammatum*, and does not develop necrosis in response to mammatoxin. However, *Saperda* species do lay eggs in branches of cottonwood and their oviposition wounds can support germination of ascospores of *H. mammatum*. Examination of these galleries for cankers reveals infections that can only be detected by magnification, as canker development is arrested at an early stage by callus formation.

Selection for Pathotoxin Resistance in vitro

The recent discovery and utilization of somaclonal variation in selection of pathotoxin resistance has been a development that has sparked increased interest in toxin-determinant diseases. For the first time, it is now feasible to do the complementary experiment to isolation of fungal and bacterial mutants lacking the ability to produce pathotoxins - namely, the isolation of variant plants resistant to pathotoxins from cell cultures initiated from susceptible donors.

The outstanding success to date in *in vitro* isolation and proliferation of pathotoxin-resistant plants was certainly a factor in the decision to employ an *in vitro* screening system in this work. However, it should be noted that the somaclonal events leading to production of pathotoxin-resistant plants appears to be primarily a phenomena of cell cultures[3]. Because lengthy culture periods were not employed in this work, the potential for somaclonal variation seems limited, but must not be excluded. The purpose of this work was not to actively pursue somaclonal variation, but the results of its utilization are mentioned here because it verifies the role of host selective toxins as determinants in disease development.

A summary of the literature on *in vitro* selection or isolation of pathotoxin resistance that has also resulted in disease resistance is shown in Table 2. In all cases, susceptible cultivars were employed as donor plants for tissue cultures. Plants regenerated from toxin-screened cultures generally exhibited a similar response to the pathotoxin and pathogen as measured against cultivars of known resistance. Often this response is not immunity, but a high degree of tolerance. In sugar cane and corn, the response was identical to the control plants, but in alfalfa and potato (*Alternaria*) some of the plants obtained exhibited resistance that was both enhanced or slightly attenuated. In potato, one out of five *Alternaria*-resistant clones derived from protoplasts lost field resistance to the disease, but some of the *Phytopathora*-resistant clones exhibited resistance to multiple races[3].

In all these cases, cultural filtrates produced by liquid culture of the fungi were used, and no attempt was made to screen with purified preparations. The protocol involved transferring established calli (as if they were being subcultured) to a medium containing a lethal or slightly sublethal concentration

of the fungal filtrate. Calli surviving this treatment were then maintained for several culture intervals on toxin-containing medium at the same or higher toxin titre. Regeneration of plants was performed on toxin-free medium.

Table 2. *In vitro* isolation of pathotoxin resistance that has afforded pathogen resistance.

Host	Pathogen	Reference
Zea mays	*Helminthosporium maydis* (race T)	82
Saccharum officinarum	*Helminthosporium sacchari*	83
Solanum tuberosum	*Phytopathora infestans*	84
	Alternaria solani[a]	85
	Fusarium oxysporum f. sp. *tuberosi*	86
Medicago sativa	*Fusarium oxysporum* f. sp. *medicaginis*	87
Brassica napus	*Phoma lingam*	88

[a]Unselected: propagules tested with toxin and pathogen.

In sugar cane and alfalfa, toxin-resistant plants could be explanted to initiate new cultures and regenerate plants that retained this resistance. In corn, progeny obtained from crosses employing toxin-regenerated plants exhibited enhanced resistance over unselected controls. In all cases, the toxin-resistant plants were unexceptional in appearance, suggesting that toxin resistance was the only trait affected. However, in alfalfa, toxin resistance from long-term selections were accompanied by polyploidy that exhibit obvious phenotypic differences.

Table 2 suggests that pathotoxin production may be common to an entire genus. Helminthosporium spp. in addition to the diseases shown, are also responsible for diseases in oats, brome grass, rice, barley, and many other forage grasses. Fusaria and Alternaria likewise contain many species (and pathovars within species) affecting a wide range of plants. In woody species, it is conceivable that this selection method could be applied to septoria canker resistance in black poplars, as a Septoria sp. causing a blight in wheat appears to employ a toxin determinant[54]. Hypoxylon rubigosum in sugar maple should also be noted in this regard.

In vitro Methods Applied to Disease Resistance in Forest Species

Diner has recently summarized the studies to date on the application of tissue culture to disease resistance in forest species[89]. The potential of in vitro technology in woody species has not been realized, principally due to the inability to regenerate plants from cell cultures. Because of this, the literature to date can be divided into two categories:

1. Studies involving culture of tissues or embryos. These investigations have been aimed at developing an in vitro bioassay for assessment of resistance or susceptibility. The advantage of tissue culture techniques in these studies appears to be the ability to investigate the host-pathogen relationship without interference from the environment or competing organisms.

2. Studies involving cell cultures. The inability to regenerate plants from cell cultures of woody species has precluded confirmation of resistance responses exhibited by cell cultures on the whole plant level. Important results have been obtained, primarily through demonstration that disease susceptibility or resistance can be expressed by dedifferentiated cells as measured by

cell growth parameters, or microscopic examination of fungal colonization of cell cultures.

Under Category 1, embryo cultures of Pinus lambertiana and P. taeda exhibited resistance or susceptibility toward Cronartium spp. similar to the natural incidence observed in the progeny groups from which they were derived[90,91]. In Populus, similar in vitro expression of resistance has been seen in cultured leaf disks of P. deltoides[92] and P. trichocarpa[54] inoculated with Melamspora medusae and Septoria musiva, respectively.

Under Category 2, the most significant result to date is the expression of resistance in embryo-derived pine callus to Cronartium ribicola, analogous to the response of in vitro culture of embryos described in the first category[93]. Two other examples merit attention as they suggest possible limitations to application of in vitro screening techniques. Suspension cultures of Ulmus americana established from clones both resistant and susceptible to Ceratocytis ulmi showed a differential growth response in the presence of cultural filtrate of this fungus. However, no differences in response to the fractionated filtrate could be detected. In addition, fungal components in the media in which suspension cultures were grown did not cause production of materials in these cells that would inhibit the growth of C. ulmi[94]. Except for changes in growth, cell suspensions apparently did not respond by producing tylosoid material, characteristic of the in vivo response. This may not be surprising in that tylose production might only be expected to occur in vascular elements and these cell types may be lacking in vitro.

Another example is examination of colonization of Castenea dentata by Endothia parasitica. Although chestnut blight resistance of the clones cultured

was positively correlated with the levels of tannins in the calli, the ability of the fungus to colonize host callus was not correlated to resistance[95]. The authors concluded that differences in callus morphology and rate of senescence altered tannin levels in a way that confounds expression of resistance. It appears that conditions arising from the tissue culture system, or alternatively clone-dependent variation in response to *in vitro* manipulation can create an artifactual situation that can make detection of resistance responses difficult. A similar explanation may account for the inability of *Cronartium fusiforme* to colonize *Pinus taeda* callus. The callus cultures were reported to produce an unknown agar-diffusible substance that was lethal to the pathogen[96].

In summary of studies on *in vitro* screening for disease resistance in woody species to date, two results encourage further application of this technique. The first result is that differential sensitivity to pathogens can be expressed by juvenile tissues (embryos) and that expression of resistance at this young age is correlated with the resistance of older plants. This implies that the tissue culture system can provide an economy of time as well as space.

The second result is that dedifferentiated cells can express resistance that is also correlated with the response of the intact plant. The current ability to produce trees from cells in the majority of commercially important forest species prevents exploitation of protoplastology and somaclonal variation as a means of inducing desirable traits that might be lacking in the genotype being cultured.

Until now, no plants have been regenerated from cell or tissue cultures of a woody species expressing tolerance *in vitro* to a selection pressure of any type.

MATERIALS AND METHODS

PLANT MATERIAL

Full-sib *P. tremuloides* crosses (designated XT-6-80, XT-4-82, XT-20-82, XT-3-83, XT-2-84, XT-3-84, XT-13-84, and XT-20-84) were made in March and April employing a cut branch technique[97]. In this procedure, floral budwood was forced in a greenhouse and hand pollinated with a small, camel hair brush. Seed was collected 21-22 days after pollination. Crosses XT-3-83 and XT-3-84 represent the same parentage and are treated as equivalent. Open pollinated *P. tremuloides* (XT-0-24-80) and *P. deltoides* (XD-0-85-72) seed was collected in mid-May and mid-June, respectively.

All branches and seed were collected from The Institute of Paper Chemistry arboretum near Greenville, Wisconsin. All seed was stored in a desiccator over $CaCl_2$ at -18°C prior to use. The material for the cross employing dead or damaged pollen was subjected to a temperature of 50°C for 3.5 minutes using a previously described pollen oven[98].

PRODUCTION OF STERILE SEEDLINGS

Seeds were sterilized by a two step procedure. In the first step, seeds were stirred with a 0.02% solution of 8-quinolinol sulfate (chinosol) for five minutes. After rinsing with sterile water (3 x 50 mL) the seeds were treated in the second step with 0.525% sodium hypochlorite (10% w/v commercial bleach) for five minutes. In both steps Tween-20 (0.4% w/v) was employed as a wetting agent.

Following a rinse with sterile water (6 x 50 mL), seeds were plated on 1% agar-water (Bacto; Difco) and incubated at 3,000 lux* and 22°C. After four days, explants were taken to establish cultures.

SHOOT DIFFERENTIATION

Cotyledons and hypocotyls were excised and placed (8 seedlings/9 cm Petri dish; 7 dishes/treatment) on a variety of media. This constituted the protocol for replicated studies of shoot differentiation within and between crosses that were subjected to a one-way ANOVA with Duncan's Multiple Range Test ($p = 0.05$). After four weeks at 22°C and 3,000 lux, morphogenesis was scored as the percentage of explants producing shoots. In addition, the number of shoots/explant with a length >1 cm was also recorded. In all subsequent work, MS media[99] containing 0.1/1.0 mg/L NAA/BA (naphthalene acetic acid/6-benzyl adenine) was employed to initiate shoot differentiation. The pH of the medium was adjusted to 5.8 prior to autoclaving and 0.8% agar was used for gelation.

To elongate the shoots and multiple buds that lagged behind in development, the explants were transferred to 1/2 MS (macro- and microelements) medium containing 0.3 mg/L BA and 1.5% sucrose. The pH and agar concentration was as above. After three to four weeks, the first 1-2 cm shoots were excised and transferred to root formation media. The remaining explants were transferred to renewed media to stimulate continued shoot formation and elongation.

ROOT FORMATION

Root formation was achieved by placing excised shoots in 1/3 MS (macro- and microelements) medium containing 0.1 mg/L IBA (δ-indole butyric acid) and 1%

*All light regimes 16/8 h; cool-white fluorescent.

sucrose. The pH was adjusted to 6.7 and 0.65% agar was used for gelation. Incubation was at 22°C and 3,000 lux. The first roots appeared as early as six days, and by three weeks plantlets were ready for transfer to soil.

PLANTLET TRANSFER

Plantlets were removed from the medium and most of the agar carefully removed from the roots. The leaves from the lower half of the stem were removed, and the plantlet was buried to this point. A soilless mixture composed of sand:peat:perlite (1:1:1) was used in Spencer-Lemaire Rootrainers (175 cc) book planters. The plantlets were watered with a systemic fungicide (Benomyl, 1 g/L) and sealed in plastic bags to maintain a humid environment. The bags containing the plantlets were kept shaded in a greenhouse for four weeks at 3,000-5,000 lux. After four weeks, plantlets were removed from the plastic bags and kept under the ambient conditions of the greenhouse.

Maintenance of Trees

Once established in soil, all *in vitro* derived trees were treated in a manner analogous to seedlings. That is, trees were transplanted to larger containers as dictated by root growth and were watered to holding capacity as required. In addition, trees were fertilized bimonthly (20-20-20; 2 g/L) and Pentac (Dienochlor; 2 g/L) was used to control two-spotted spider mites at regular intervals.

HYPOXYLON MAMMATUM STOCK CULTURES

Two mass isolates of *Hypoxylon mammatum*, designated 257-6-10 and 257-6-41 were generously provided by M. Ostry of the U.S. Department of Agriculture, North Central Forest Experiment Station, St. Paul, MN. The isolates were

obtained in 1980 from cankered tissue associated with insect (cicada) wounds. Both isolates came from the Oconto River Seed Orchard in the Nicolet National Forest, WI.

Stock cultures were maintained at 20°C in the dark on 2% malt extract (Difco) solidified with 1.5% agar (Bacto; Difco). One of the isolates (257-6-41) rapidly developed a dense mycelial mat that required subculture every two weeks. The other isolate was transferred at monthly intervals. All toxin preparations used were from isolate 257-6-41 unless otherwise indicated.

TOXIN PRODUCTION

Mammatoxin was produced by liquid culture of Hypoxylon mammatum after the method of Griffin[100] and Stermer[73]. Mycelial plugs (8 mm) were blended in 25 mL of sterile water by the action of broken glass to form a brei of 2 plugs/mL. This was used to inoculate a modified Fries medium[101]; (Appendix, Table 22) to give a final density of 0.1 plug/mL. Following rotary incubation (100 rpm) at 28°C for 18 days in diffuse light, the mycelia were filtered through cheesecloth and manually wrung to remove most of the entrained filtrate. The filtrate was further clarified by passage through filter paper (Whatman No. 1; qualitative) and the volume noted. This volume formed the basis for all further manipulations.

After concentration on a rotating evaporator to 10% of the initial volume, an equal volume of methanol was added, and after standing overnight at -22°C, a precipitate was filtered. The filtrate was evaporated to dryness, and the residue dissolved in an amount of water equivalent to 2.5% of the initial filtrate volume. This toxin solution was employed in the initial in vitro screening and seed germination studies.

For the majority of screening and biological assays the toxin solution was modified by an extraction procedure of Griffin[100]. The solution (2.5% concentrate) was basified (pH = 11) with 10M KOH, and partitioned against three equal volumes of ethyl acetate. The aqueous phase was subsequently acidified (pH = 2.5) with 12M HCl and extracted with three equal volumes of ethyl acetate, followed by three equal volumes of water-saturated n-butanol. Along with the aqueous remainder, the three extracts were concentrated to dryness and dissolved in water representing 2.5% of the initial filtrate volume. Each of these four fractions, designated basic ethyl acetate (OH^- EtOAc), acidic ethyl acetate (H^+ EtOAc), acidic n-butanol (H^+ n-BuOH) and the aqueous remainder, were tested independently for toxic activity. In addition, a blank employing Fries medium was taken through the same concentration and extraction procedure.

CHROMATOGRAPHY OF MAMMATOXIN

Gel-Filtration Chromatography (Bio-Gel P-2; Bio-Rad; 200-400 mesh) of the toxin containing extracts (H^+ EtOAc and H^+ n-BuOH) was performed after the method of Griffin[100] and Schipper[64]. The column volume was 66 mL, the temperature was 4°C, and water was used as an eluant at a flow rate of 0.1 mL/min. For each extract, 2.5 mL was applied to the column and the elution profile monitored by UV absorbance (280 nm). No toxin containing material eluted at less than the void volume (~ 22 mL) and the fractions were pooled based on the absorbance profile. Each of the pooled fractions was evaporated to dryness, and taken up in 2.5 mL of water and tested for toxic activity in the leaf puncture bioassay.

MAMMATOXIN BIOASSAY

Whole Leaf Bioassay

The first bioassay employed was the original assay described by Schipper[64]. In this procedure, the two toxin containing extracts (H^+ EtOAc and H^+ n-BuOH) were reconstituted in the ratio of 1:1:2 (H^+ EtOAc:H^+ n-BuOH:H_2O). This solution was used to fill microcentrifuge tubes (250 µL) that served as reservoirs for the assay.

Three fully expanded, unblemished leaves from each tree were excised at the point where the petiole is attached to the stem, and immediately placed in the filled reservoirs. Following incubation in a humidified chamber for 48 hours at 28°C, the bioassay was scored as the percentage of the leaf blade area that had developed necrosis. A dot grid (6.25 mm^2/dot) was employed to measure the total leaf area and the extent of necrosis.

Leaf Puncture Bioassay

The leaf puncture bioassay employed was after the method of Griffin[100], Stermer[73], and Bruck and Manion[74]. Three unblemished leaves, one of which was less than fully expanded, were excised as above and the petioles placed in vials containing 1.5-3.0 mL of water. The leaf blade was carefully rinsed with water and wiped dry. A minutin insect pin (o.d. = 0.5 mm) was used to create puncture holes, which were inoculated with 3 µL drops of various toxin preparations. After incubation as above, the bioassay was scored as lesion diameter to the nearest 0.5 mm.

Unless otherwise stated, all leaf puncture bioassays were performed by dilution of the 2.5% concentrate of the H^+ EtOAc and H^+ n-BuOH extracts with water (1:1) and each extract was tested separately. Each fraction was tested on

a respective half of the leaf, as divided by the midvein. Three puncture holes/extract/leaf for a total of nine determinations/extract/tree formed the basis for statistical analysis by a one-way ANOVA ($p = 0.05$) with Duncan's new Multiple Range test. In this way, each tree became a treatment, each leaf became a replication, and each lesion became a subsample within a replication.

IN VITRO EXPERIMENTS WITH MAMMATOXIN

Isolation and Micropropagation of Mammatoxin-Resistant Aspen

Initially, the crude filtrates of isolates 257-6-10 and 257-6-41 concentrated to 2.5% of the initial volume were employed at various levels. However, for the majority of the screening work the H^+ EtOAc and H^+ n-BuOH extracts (from isolate 257-6-41) were used after they were mixed in equal proportions. The combining of these two extracts effectively represented screening with a preparation representing 5% of the initial filtrate volume. Thus, to put all screening on an equal basis, the concentrations shown for screening with the crude, nonextracted filtrate should be doubled.

In all cases, the toxin preparation was adjusted to pH = 5.8, filter sterilized, and added to shoot proliferation medium that had been cooled to 40°C. The toxin preparation was substituted for part of the water in the medium, so that a constant medium concentration was maintained.

After four weeks, morphogenesis was scored and explants exhibiting multiple bud formation were rescued by transfer to shoot elongation medium lacking toxin. All subsequent manipulations to obtain plants were done in the absence of toxin.

Mammatoxin Screening with Crude Filtrate

XT-0-24-80

In the first application of mammatoxin to the shoot proliferation medium, the 2.5% concentrate from isolate 257-6-10 was substituted at levels of 10 and 25% (v/v). In addition a control was run with noninoculated Fries medium concentrated to 2.5% of the standard volume. Four seedlings (4 hypocotyls; 8 cotyledons) were used at each level. This was the only in vitro work performed with isolated 257-6-10.

XT-3-83

In this screening, the 2.5% concentrate of isolate 257-6-41 was used at levels of 0, 2, 4, and 8%. In addition, cottonwood (XD-0-85-72) was run synchronously at the same levels. Eight seedlings per plate and three plates per toxin level were employed in each screening.

Germination of Seeds on Toxin-Containing Medium (XT-0-24-80 and XT-3-83)

Seeds were sterilized in the described manner, but were plated on agar-water substituted with 0, 8 and 33%, and, 0, 4 and 8% of the 2.5% concentrate for XT-0-24-80 and XT-3-83, respectively. After seven days, germination was noted and surviving seedlings were explanted on shoot proliferation medium containing 0 and 8%, and, 0, 2 and 4% of the above toxin preparation for XT-0-24-80 and XT-3-83, respectively.

Mammatoxin Screening with Extracted Filtrate

As noted previously, the remainder of the in vitro screening was performed with two toxin containing extracts (H^+ EtOAc and H^+ n-BuOH) combined in equal proportions prior to addition to shoot proliferation medium. Screening with

this preparation was always performed with cotyledonary explants (20 cotyledons/plate) and three plates/treatment. The controls (no toxin and cottonwood) were always run synchronously with the same protocol.

To ascertain the effect of toxin concentration on shoot proliferation efficiency, seedlings from crosses XT-2-84 and XT-3-83 were explanted on organogenesis medium containing 0, 1, 2, 4, 6, and 8% toxin. After four weeks, survivors on toxin-containing medium were rescued and propagated, with all tissue culture propagules retaining the label of the initial explant to ensure clonal grouping. From each treatment, explants producing callus only (5 calli/plate) were blotted dry and weighed to the nearest 0.1 mg.

In Vitro Bioassay

Cotyledons from seedlings from crosses XT-3-84 and XT-20-84 were plated on shoot proliferation medium containing 2 and 4% toxin, and 4% toxin, respectively. The remainder of the seedling (lacking one cotyledon) was transferred to a soilless mixture containing equal proportions of peat, vermiculite, and perlite (40 seedlings/26 cm saucer). The saucers were covered with a glass plate to maintain high humidity. After four weeks, in vitro organogenesis was scored, and the survivors were rescued, proliferated, and established in soil. Single-cotyledon seedlings corresponding to morphogenic cultures were segregated from the population and transplanted to the soilless mix.

In this way, the in vitro mammatoxin response of tissue culture propagules derived from toxin-resistant cultures can be compared with seedlings from which they were explanted. A similar approach was used to establish a control population (XT-3-83) of 24 seedlings and six tissue culture derived "ramets" from each seedling.

RESULTS AND DISCUSSION

PLANT MATERIAL

Parent trees in all crosses were chosen with the stipulation that they be readily available and have a database on Hypoxylon canker incidence (see Table 3).

Table 3. Parentage of Populus material.

Cross[a]	Parentage ♀	Parentage ♂	Hypoxylon Rating[b]	Germination, %[d]
XD-0-85-72	D-8-65	open	—	88
XT-6-80	T-20-56	T-46-60	M	--
XT-0-24-80	T-5-61	open	MH	93
XT-4-82	T-53-60	T-44-60	MH	--
XT-20-82	T-1-59	T-44-60	MH[c]	78
XT-3-83	XT-22-56,S2	T-44-60	M	69
XT-2-84	T-24-60	T-13-58	H	98
XT-13-84	T-1-58	T-6-61	M	95
XT-20-84	T-1-58	T-20-60	H	98

[a]X = experimental cross, D = P. deltoides, T = P. tremuloides, o = open-pollinated.
[b]Minimum 10-year rating, based on system used by Project 3537 (M = medium, MH = medium-high, H = high).
[c]Heat-treated pollen; rating based on female tree.
[d]After sterilization except XT-13-84.

Field data on canker incidence was of two forms: direct observation of earlier planted progeny groups (represented by repeat crosses XT-4-82, XT-2-84, XT-13-84, and XT-20-84) or indirect determination of a given parent by its performance in a range of monitored progeny group plantings. The indirect

determination was less desirable but was necessary for XT-6-80 and XT-3-83 which had never been planted and for XT-0-24-84 and XT-20-82 in which the male was unknown or "dead" (heat-treated pollen).

In all cases, at least a 10-year-old measurement of canker incidence was available, and in some crosses (XT-2-84, XT-13-84, and XT-20-84) 20-year data were available. These periodic measurements have been condensed into a rating system based on the commonly accepted annual infection rate of 1-2%/year. For example, at age 10, a low rating is considered 0-5%; medium, 6-10%; medium-high, 11-15%; and high, +16%. These percentages were calculated by taking the number of trees that had died from hypoxylon canker plus the number of trees with observed infections and dividing by the total number of trees involved in the observation.

According to the above rating system (a "range" of canker incidence is depicted in Table 3) the conspicuous absence of crosses with low ratings should be noted. That the accepted annual infection rate spans the range encompassing a medium to high rating suggests that there may be little actual difference in hypoxylon susceptibility in all the crosses employed.

The heat-treated pollen cross (XT-20-82) was originally considered as a means of ensuring variation in the response of a progeny to mammatoxin. Populus species have been shown to undergo parthenogenesis when pollinated with dead pollen[102]. While heat treatment of pollen by this method has never been reported to give rise to the haploid condition, it did produce a significant number of seedlings with aneuploidy or mixoploidy. The mixoploid condition is particularly intriguing because integral chromosome numbers of the haploid state (1n = 19) presumably could only have arisen by endoreduplication. Homozygous diploids

obtained in this way might be desired for their ability to "breed true," in this case for canker resistance. In any case, a fractional or integral change in chromosome number might be expected to be reflected in a phenotypic variation in the response to mammatoxin.

Surprisingly, seed set and germination were not adversely affected by the use of heat-treated pollen. Seeds that germinated were indistinguishable from those obtained from conventional crosses. This was notable because the smaller seed (50 mesh) was used rather than the usual 40 mesh. In aspen, pollen grain size has been related to ploidy level[103] and it was thought that the smaller seed would more likely be products of a parthenogenic event.

STERILE SEEDLING PRODUCTION

The seed sterilization conditions were optimized with respect to germination. Employing higher concentrations of sodium hypochlorite and/or longer time intervals had adverse affects on germination and seedling vigor. The most typical indicator of harsh sterilization conditions was severely attenuated seedling root development.

To ensure asepsis, seeds must be adequately "wetted" during sterilization and seedlots should be relatively free of bract. Aspen seeds are quite buoyant and the first step of the sterilization procedure is a pretreatment to prevent flotation.

Bract was only a problem in seedlots zealously collected. Bract and debris associated with seed capsules is particularly difficult material to sterilize without harm to the seedlings, and screening is recommended for crosses in which this is a problem.

SHOOT PROLIFERATION

Multiple adventitious bud formation has previously been shown to be under hormonal control for a variety of Populus organ and cell culture systems. A high cytokinin-to-auxin ratio has been used to proliferate shoots from axillary buds[6] of P. tremuloides (BA/NAA = 25), and a ratio of 10 (BA/IBA) has been used with plated cell suspensions of P. tremula[104].

To further examine the hormonal ratio and absolute levels for shoot proliferation from seedling explants, a limited study of the effect of growth regulators on morphogenesis was performed. Organogenesis frequency (% of explants) and the number of shoots per explant were measured under various combinations of NAA and BA. The number of shoots per explant was not entirely indicative of the proliferative ability of the explant, as many buds had not elongated at the time of scoring in some treatments. The results of this study are depicted in Table 4. In general, a greater percentage of hypocotyl segments gave rise to multiple adventitious buds, and the number of buds/explant obtained was more uniform than for cotyledonary explants.

The effects of growth regulators can be divided into two groups based on the cytokinin regimes. The lower level of BA (0.1 mg/L) was characterized by production of one to three shoots that appeared without visible callus formation a week after the cultures were initiated. This response was relatively insensitive to the auxin levels employed, but at the higher level (0.1 mg/L NAA) extensive root formation replaced shoot differentiation.

The higher level of cytokinin (1.0 mg/L) resulted in cultures that proliferated callus which subsequently differentiated shoots after three to four weeks. The number of shoots obtained was similar for the lower levels of auxin

(0.01 and 0.1 mg/L), but at 1.0 mg/L NAA extensive parenchymatous callus formation predominated instead of the smooth, nodular callus that gave rise to shoots. This effect was clearly seen when loblolly pine medium (LM, 105) was employed. At 1.0/1.0 mg/L NAA/BA the shoot induction frequency dropped significantly for both explants on LM when compared to MS and callus formation occurred almost exclusively from cotyledons. Although callus formation dominated these treatments, it was not at the expense of bud differentiation. Transfer of these cultures to MS medium containing 0.1 mg/L NAA and 1.0 mg/L BA gave rise to clearly discernible shoots within two weeks. This suggests that bud differentiation was taking place on LM containing 1.0 mg/L NAA and BA, but at a much slower rate.

Table 4. Effect of media and growth regulator regime on shoot proliferation characteristics of seedling explants (XT-3-83).

Medium[z]	NAA/BA, mg/L	Ratio (BA/NAA)	Shoot Induction, %		Shoots/Explant	
			Hypocotyls	Cotyledons	Hypocotyls	Cotyledons
MS	0.01/1.0	100:1	95[a]	47[a]	6.1[b]	4.4[a]
	0.1/1.0	10:1	96[a]	80[a]	5.9[b]	4.8[a]
	1.0/1.0	1:1	61[b]	48[b]	3.1[c]	3.1[b]
	0.01/0.1	10:1	65[b]	29[c]	1.5[d]	1.7[bc]
	0.1/0.1	1:1	50[b]	15[cde]	1.1[d]	2.0[bc]
LM	0.01/1.0	100:1	84[a]	20[cd]	6.8[d]	5.0[a]
	0.1/1.0	10:1	95[a]	19[cd]	7.3[a]	4.4[a]
	1.0/1.0	1:1	31[c]	4.6[de]	3.5[c]	1.4[bc]
	0.01/0.1	10:1	66[b]	5.4[de]	1.3[d]	1.6[bc]
	0.1/0.1	1:1	32[c]	0.86[e]	1.0[d]	0.14[c]

[a,b,c,d,e]Within a column, means followed by a common superscript are not significantly different by one-way ANOVA with Duncan's Multiple Range Test ($p = 0.05$).

[z]Murashige and Skoog (MS), 1962[99]; Litvay et al. (LM), 1982[105].

In contrast, when LM was compared to MS at the two growth regulator levels that gave rise to the greatest bud proliferation (0.01/1.0 and 0.1/1.0 mg/L NAA/BA), there was little difference between the two media. Although MS and LM produced comparable numbers of shoots at the time of evaluation, MS containing 0.1/1.0 mg/L NAA/BA was used in all subsequent work as it consistently produced the greatest number of buds that could subsequently be elongated.

SHOOT ELONGATION

Transfer of cultures exhibiting multiple buds to 1/2 MS medium containing 0.3 mg/L BA stimulated elongation of those buds that had lagged behind in development (see Fig. 1). In addition, this treatment caused axillary bud elongation in existing shoots which could also be excised and rooted. By taking care during excision to leave a portion of the shoot behind, "microcoppicing" became an effective way to perpetuate certain seedlings in a juvenile state. Under these conditions, full-sib clones were established in which a varying number of ramets (1-60) had been rooted.

Figure 1. Shoot culture established on elongation medium (8 weeks).

Although mass clonal propagation was not an objective of this work, it became apparent that within each cross examined, a portion of the cultures (5-20%) would supply a continuous harvest of shoots at regular intervals. The cultures that exhibited this potential were proficient both at production of adventitious shoots at the cut surface of an excised shoot and by precocious elongation of axillary buds on preexistent shoots.

The combined multiplication factor of these two processes operating together was highly variable, but averaged about three to five. While the lack of efficiency in axillary bud elongation was limited by the number of buds existing on these short shoots, production of adventitious buds occurred in an analogous fashion to the low cytokinin treatments of seedling explants; only a few shoots with very little callus formation were observed. Cytokinin was a requirement for both morphogenic processes, as shoot cultures could not be established on hormone-less media.

In this micropropagation method, the cultures rapidly became dominated by those buds that rapidly developed into shoots. From these relatively few initials (see Table 4) axillary bud elongation provides the majority of the multiplication. The limited number of primary differentiational events represented in this scheme may preclude isolation of low frequency mutational events that might be captured if a cell-by-cell evaluation was occurring. However, genetic stability is presumably assured by basing the micropropagation scheme on existent axillary buds[106]. Deviations from the true type in this system might be expressed by blocks of ramets within a clone, and not by a single individual.

ROOT FORMATION

Root formation was facile, apparently due to the juvenile nature of the material, as woody cuttings of quaking aspen are considered difficult to root[107]. The ease of rooting made evaluation of treatments difficult. All media formulations tested (Hoaglund's solution 1/10 and 1/3 strength; MS media; 1/3 strength ± 1% sucrose) and all IBA concentrations (0, 0.1, and 1.0 mg/L) supported root formation and produced plantlets that were successfully transferred to soil. Media formulations were evaluated subjectively by the appearance of the steckling and not by the frequency of rooting. The criterion of appearance was based on the resemblance of the in vitro-derived root system to that of a seedling. Hence, a "good" rooting treatment was one that produced thick, vigorous initials exhibiting lateral branching and a profusion of root hairs along primary and secondary roots.

The most important factor in obtaining a root system with a natural appearance was the inclusion of a carbon source (1% sucrose) in the medium. While the benefits of sucrose supplementation quantitatively enhanced root formation frequency (see Fig. 2), the appearance of the root system was markedly different than those from treatments lacking sucrose. Formulations lacking sucrose often produced comparable numbers of initials, but roots were thin, unbranched, and root hairs were conspicuously absent. Furthermore, root morphology was not affected by the concentration or presence of IBA.

Additions of IBA (0.1 mg/L) to 1/3 MS medium supplemented with 1% sucrose tended to increase the rate of root formation, but had no effect on the overall appearance of the root system. However, increasing the IBA level to 1.0 mg/L caused the shoots to rapidly differentiate a large basal mass of callus that

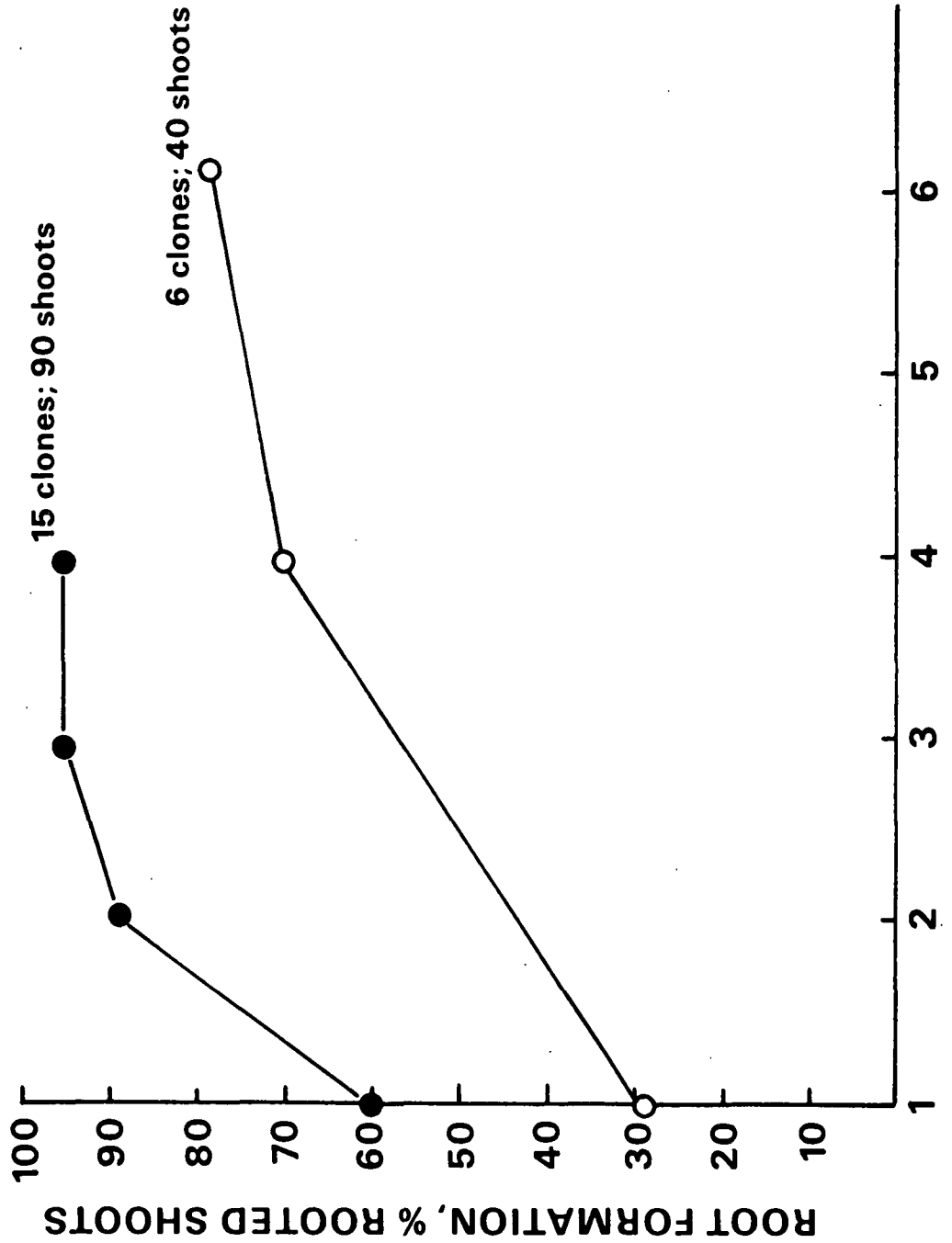


Figure 2. Effect of sucrose addition (open circles = no sucrose; closed circles = 1% sucrose) on root formation (XT-0-24-80; 1/3 x MS + 0.1 mg/L IBA).

later differentiated roots. Because this morphology is not analogous to root development in seedlings, the higher level of IBA was not used in subsequent treatments, although it was noted that this treatment produced plantlets successfully transferred to soil.

PLANTLET TRANSFER

Figure 3 shows a plantlet representative of those readily established in a simple soilless potting mix and hardened-off without the need of growth chambers or intermittent misting. Survival approached 80-90%, but on several occasions was reduced to 10-20%. Transfers with high mortality were due to equipment failure and lack of fungi control.

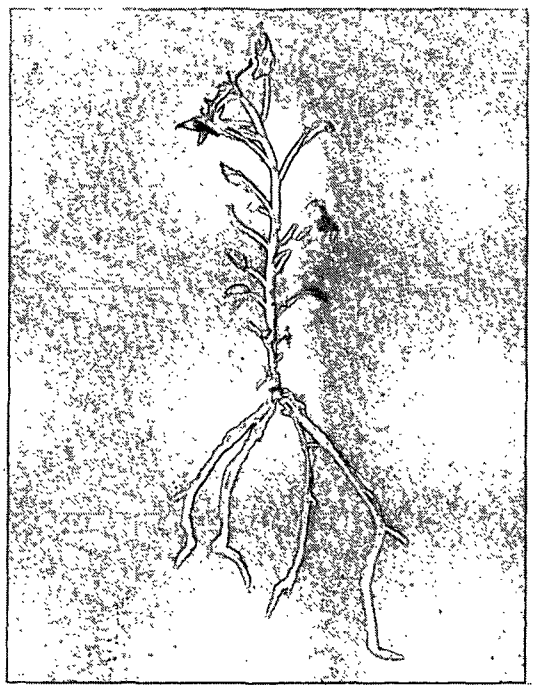


Figure 3. Rooted plantlet ready for transfer to soil.

At the time of transfer, a number of plantlets exhibited characteristics that appeared to be artifacts of the tissue culture system (i.e., vitrified or lanceolate leaves, or a rosette pattern of the terminal bud). However, once established in the ambient conditions of a greenhouse, leaf and growth habit developed in a manner virtually indistinguishable from quaking aspen seedlings (see Fig. 4). In addition, tissue culture propagules exhibited growth rates similar to seedlings after establishment in soilless medium (see Fig. 5).

GENETIC VARIATION IN IN VITRO MORPHOGENIC BEHAVIOR

Examination of the organogenesis behavior of several crosses (see Table 5) revealed a genetic component to in vitro morphogenesis. The variation between seed lots was primarily in the ability to differentiate multiple buds and their subsequent ability to elongate and proliferate as shoot cultures. Once rootable shoots were obtained there was little variation in rooting frequency within or between single explant clones from each cross. In turn, this served to make survival in soil uniform between the progeny groups examined. The link between root system appearance and survivability was evident in XT-20-82 - the cross employing heat-treated pollen. In this cross, all the single explant clones had the capacity to produce roots but did so less often or produced stunted, knobby roots. In these cases, survival was zero and two of the five clones could not be established in soil.

The use of seedling explants further implies that another level of variation will arise due to recombination and segregation during the meiotic steps preceding zygote formation. Differences between the morphogenic response of individual seedlings explanted on shoot proliferation medium is demonstrated by the presence of explants that differentiate parenchymatous callus in contrast to

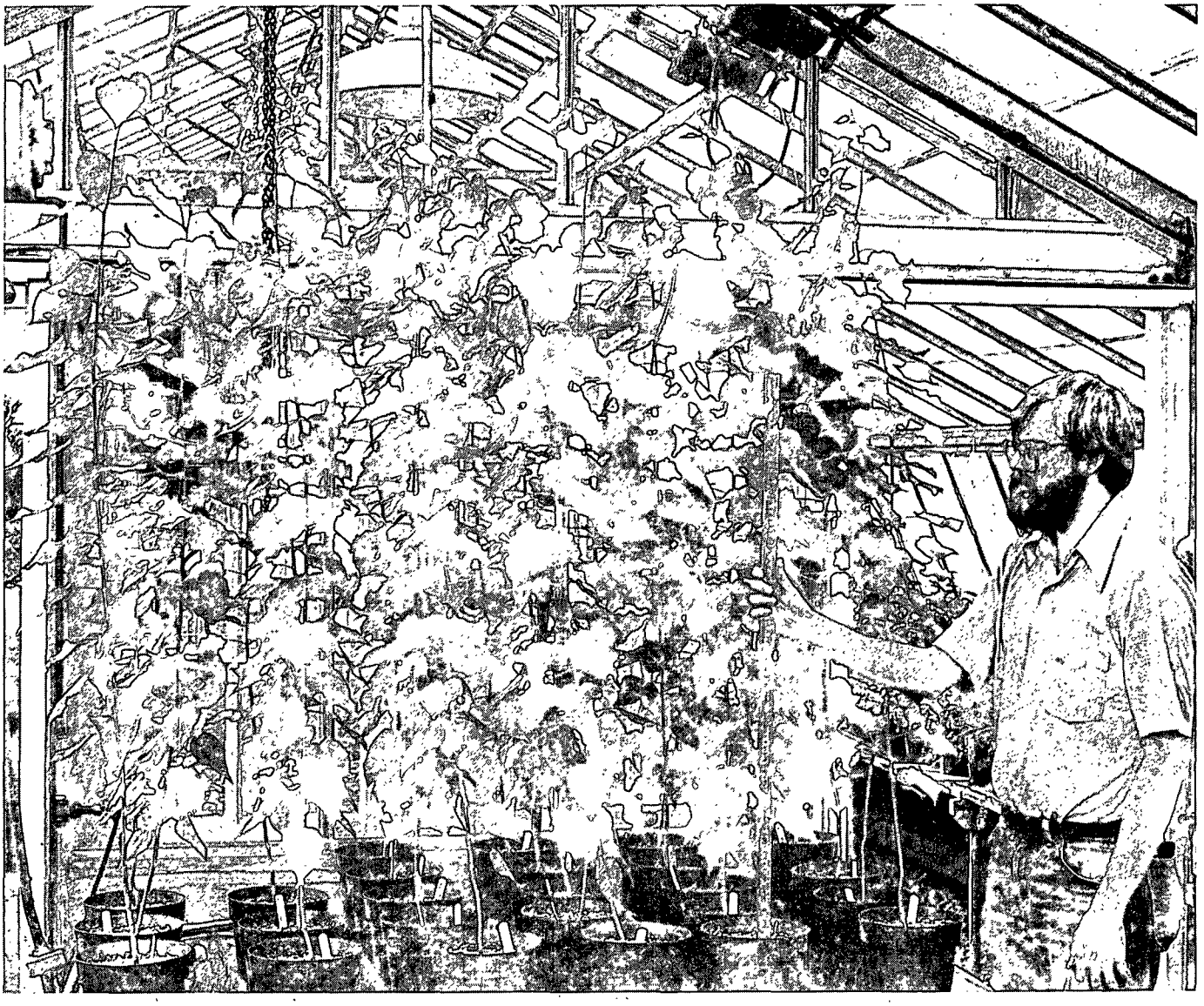


Figure 4. Tissue culture-derived trees 14 weeks after establishment in soil-less medium.

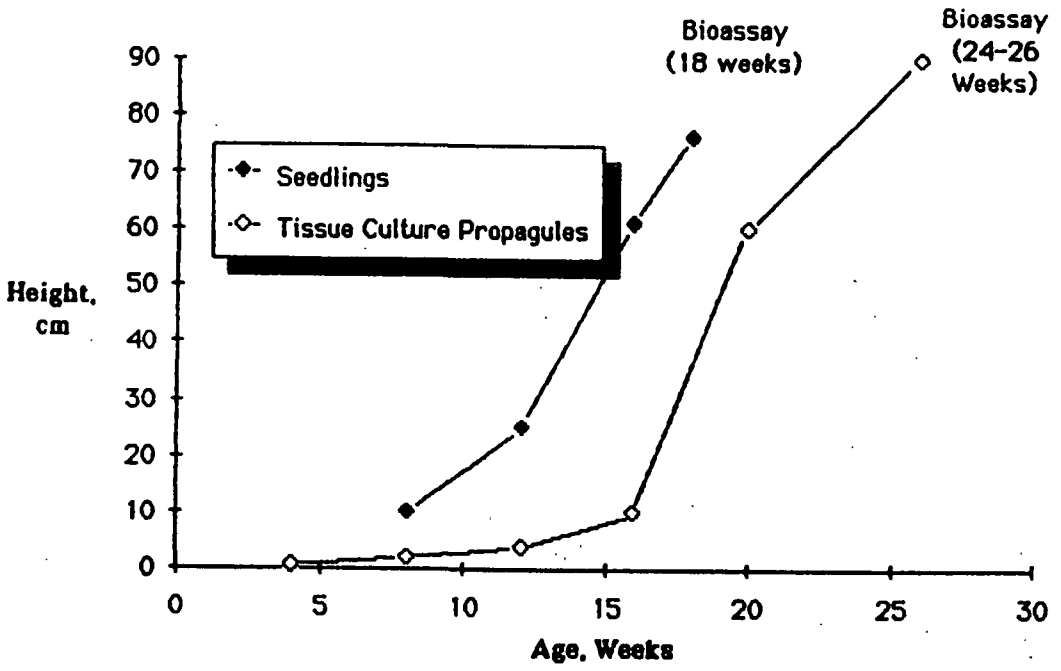


Figure 5. Height growth vs. age for seedlings and tissue culture propagules (XT-3-83). For the propagules, bud initiation, 0-4 weeks; shoot elongation, 4-7 weeks; root formation, 7-10 weeks; transfer to soil, 10-14 weeks; open growth in green house +14 weeks.

the convoluted, nodular callus that gives rise to buds. In most seedlots, one of these calli types dominated the development of a particular explant but both calli types were routinely present on the same culture. This situation was especially evident in XT-2-84, where bud differentiation was concomitant with callus formation. The propensity of this cross to differentiate callus led to not only lowered shoot induction frequency, but a reduction in the absolute number of buds as well. Curiously, this was the same type of behavior that occurred when the auxin level of the shoot proliferation medium was raised to 1.0 mg/L in the culture of explants from XT-3-83. This would seem to suggest that any genetic component to in vitro morphogenic potential may not be absolute. Instead, certain genotypes within and between crosses may have different sensitivities to physical, nutritional or hormonal conditions of the tissue culture

environment, which by adjustment, could yield the desired pattern of differentiation. Certainly in aspen, where hormonal control of differentiation has been demonstrated[6,11,12], hormonal manipulation would seem more attractive than nutritional or physical adjustments.

Table 5. Organogenic behavior of four different aspen crosses.

Cross No.	Shoot Induction Frequency, %	Root Formation Frequency, %	Root Formation Frequency; Clones, %	Root Formation Frequency; Ramets, %
XT-3-83	80[a]	84 (51)[x]	96 (43)	94[y] (43)
XT-0-24-80	69[a]	75 (56)	94 (42)	88 (42)
XT-2-84	51[b]	13 (60)	75 (8)	84[y] (6)
XT-20-82[w]	39[b]	23 (22)	100 (5)	60 (5)

[a,b]Within this column, means followed by a common superscript are not significantly different by one-way ANOVA with Duncan's Multiple Range Test ($p = 0.05$).
[w]Heat-treated pollen cross.
[x]Value in parentheses indicates the number of clones for which the frequency was determined.
[y]Minimum 6 ramets/clone.

In addition to differences in shoot induction frequency, the ability of buds to develop into rootable shoots also varied between seedlots. In particular XT-20-82 and XT-2-84 developed a high percentage of leafy buds that never elongated and developed stems. This situation appeared analogous to shoot elongation in P. deltoides (cottonwood) organ cultures employed as a control in toxin-containing treatments. Although cottonwood hypocotyls were more capable of shoot differentiation than cotyledons, cultures of both organs differentiated multiple buds that could not be elongated under any of the treatments examined, (1/2 MS + 0.3 mg/L BA; 1/2 MS; shoot differentiation medium). The critical factor in cottonwood and the above seedlots might be in the duration of the

shoot induction treatment. In this regard, cytokinin pulses of two weeks have been shown to enhance shoot development over four week treatments in Pinus taeda L. despite the similar number of buds differentiated in both treatments[108].

As noted previously, some explants in each cross were highly capable of elongation of shoots and axillary buds on those shoots, allowing shoot cultures to be established in these cases.

All single explant clones that produced elongated shoots could be divided into two categories based on rootability. Clones established from each cross either produced ramets that rooted at the frequency shown, or failed to root at all.

That there exists an apparent genetic component to in vitro morphogenic capability is a concept as new as the field of plant tissue culture. In retrospect, it is not surprising that this component exists, as it has an in vivo analogy. Indeed, for species propagated vegetatively, differences in morphogenic capacity between cultivars are ubiquitous and have been known in some cases for centuries. Two outstanding examples are represented in Populus - the success of the antediluvian P. nigra cv. italica (Lombardy poplar) and, in this century P. euramericana cv. I-214 can both be attributed to the capacity for propagation through rooting of cuttings[109]. In aspen, clonal variation in in vitro bud differentiation[6] has been previously demonstrated.

It is significant to note that XT-2-84, a normal diploid cross, exhibited a morphogenic behavior in vitro very similar to the cross produced with heat-treated pollen (XT-20-82). While chromosome numbers were not determined, the abnormal appearance of the roots from XT-20-82 tissue culture propagules

suggests that the heat treatment caused an alteration that was expressed *in vitro*. That conventionally produced material could respond as if it had been "damaged" implies that a considerable range of different genetic sources should be examined prior to *in vitro* manipulation. In particular, if the success of an *in vitro* screening program depends on obtaining plants, sources should be chosen with a high regenerative capacity to increase the likelihood of expression of the desired trait. In the case of XT-20-82, only three single explant clones (10 ramets) were ultimately established in soil from 120 explants cultured for a multiplication factor of 0.083. Although the use of dead pollen may be a method for obtaining homozygosity for mammatoxin resistance, it may also be quite difficult to obtain plantlets from cultures of this type of cross even though they may exhibit *in vitro* resistance.

EXPERIMENTS RELATED TO MAMMATOXIN

Mammatoxin Production, Isolation, and Bioassay

Mammatoxin was routinely produced in three liter batches, providing 60-70 mL of the 2.5% crude filtrate concentrate. In the inoculation brei, it was important to have the mycelia well blended. This in turn was a function of the sharpness of the broken glass used in the homemade blenders.

Owing to the lack of structural information on mammatoxin, there is no analytical method for determining its absolute concentration in the filtrate. Absorbance at 240 nm of toxin preparations fractionated by HPLC has been weakly linked to the leaf puncture bioassay[73]. The absorbance spectrum of the crude or extracted filtrate employed contained too many interfering substances to permit the use of this technique. Analytical determination has been further compounded by the observation that mammatoxin is a collection of molecules that vary in

concentration over the course of liquid incubation of the fungus[73]. In an effort to maintain consistent toxin preparations from batch to batch, inoculation, incubation, and filtrate evaporation and extraction methods were carried out under as close to identical conditions as possible.

In actuality, these efforts to maintain equal toxin concentrations between batches were sufficient to produce a toxin preparation that was invariant in the response it elicited from batch to batch (see Table 6). These preparations caused an identical response in sensitive clones due to independence of lesion diameter at high relative mammatoxin concentrations (see Fig. 6). For the collection of susceptible clones shown, lesion diameter increased in direct proportion to relative mammatoxin concentration up to some titre at which lesion size no longer increased. This type of response is characteristic of host-selective toxins[83] and results in a constant bioassay result over a high, but considerably wide range of toxin concentrations. For the clones examined (except clones 1 and 80, H^+ n-BuOH), insignificantly different lesion diameters were obtained over at least an eightfold dilution of the preparations tested (64-8, Fig. 6). Therefore, any changes in inoculation density or filtrate concentration and extraction volumes would have to be substantial (and presumably detectable) in order to alter the result of the bioassay. The duration of the incubation period must still be strictly adhered to in order to avoid alteration in the type and/or concentration of toxin. The time period was set at 18 days, as it was coincident with the development of a dark brown color to the liquid-grown cultures.

Dilution of the toxin preparations provided further insight into qualitative differences between the preparations employed. When tested at the standard level used in most of the leaf puncture bioassay work, both extracts appeared

comparable in toxicity. With dilution, it appeared that the n-butanol extract was less toxic than the ethyl acetate extract as the necrotic response disappeared at higher concentration. An appreciation for the sensitivity of these clones to mammatoxin can be gained when it is considered that resistant individuals failed to form lesions at the highest levels tested. These individuals tolerated toxin levels that were in some cases (clones 8 and 49) in excess of 128 times more concentrated than preparations that produced necrosis in sensitive clones.

Table 6. Effect of different H. mammatum filtrate preparation on leaf puncture bioassay[w].

Preparation Date		Lesion Diameter, mm					
		H^+ EtOAc			H^+ n-BuOH		
	Clone	84-3	84-4	107S-4	84-3	84-4	107S-4
5/10/84		6.0[a]	6.3[a]	3.4[a]	6.0[a]	4.8[a]	1.8[a]
1/8/85		7.2[a]	6.2[a]	5.7[a]	5.5[a]	4.7[a]	3.3[a]

[a]Within a column, means followed by a common superscript are not significantly different by one-way ANOVA with Duncan's Multiple Range Test ($p = 0.05$).
[w]Tissue culture propagules, XT-3-83; extracted filtrate 257-6-41.

An extraction procedure appeared desirable because of problems with the initial solution employed as a control for in vitro shoot differentiation. When the uninoculated Fries medium was concentrated to 2.5% of its initial volume and added to shoot proliferation medium at various levels (see Table 7), it also inhibited bud initiation, but was not lethal to the cultures. When it is considered that concentrated Fries medium contains high concentrations of various macro- and microelements (e.g., 20% sucrose, 1M potassium glutamate), it does not seem unreasonable to assume constituents of the fungal medium could be deleterious to organogenesis when added to shoot proliferation medium. Uninoculated

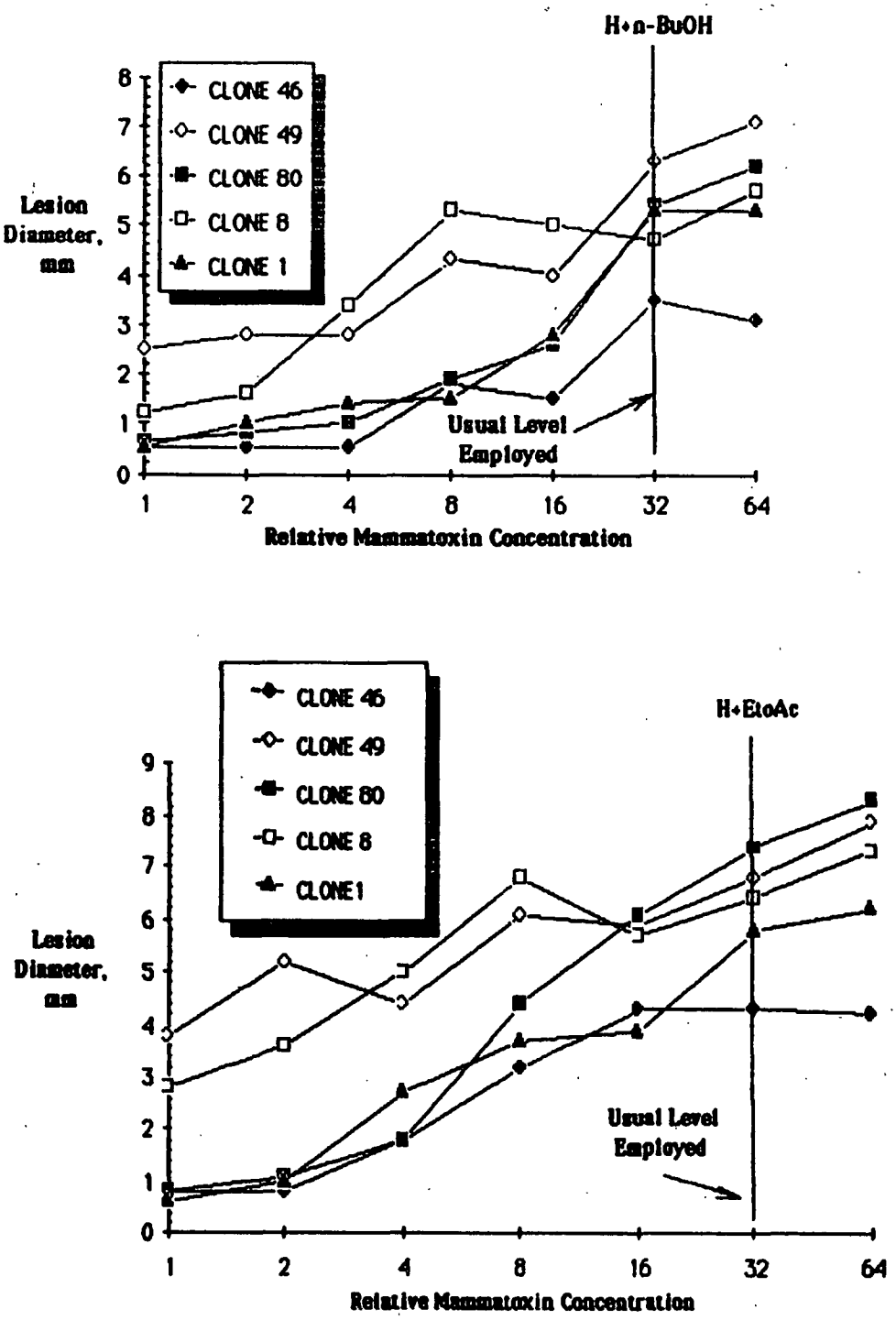


Figure 6. Effect of extract dilution on lesion size obtained in the leaf puncture bioassay for several clones (XT-3-83).

Fries medium is also questionable as a control, given that growth and metabolism of the fungus not only alters the original constitution of the medium, but introduced additional compounds that could alter morphogenesis of tissue cultures in a manner unrelated to the effect of the phytotoxin.

Table 7. Mammatoxin screening (isolate 257-6-10) of XT-0-24-80.

Treatment[a]	Explant	Callus Formation, %	Shoot Induction Frequency, %	Shoots/Explant
Control	Cot	94 ± 0	68 ± 6	6 ± 3
	Hypo	100 ± 0	87 ± 13	6 ± 2
10% Fungal filtrate	Cot	14	14	2
	Hypo	50	50	12.5
10% Fries medium	Cot	100	13	1
	Hypo	100	38	8
25% Fungal filtrate	Cot	0	0	0
	Hypo	0	0	0
25% Fries medium	Cot	0	0	0
	Hypo	0	0	0

[a]Inoculated and uninoculated Fries media concentrated to 2.5% of the initial volume prior to formulation.

By taking advantage of the host selectivity of mammatoxin, closely related, nonhost organisms can be employed as controls to directly examine the effect of mammatoxin on similar processes that would occur in the host. In this work, a control species was required that would permit the evaluation of the effect of mammatoxin on *in vitro* (morphogenesis of tissue cultures) and *in vivo* (bioassay of intact plant organs) processes.

Populus deltoides (eastern cottonwood) was chosen for a control species as it met both of these criteria. The incidence of hypoxylon canker on cottonwood

is rare, and inoculation of artificial wounds with mycelia fails to produce cankers[28]. Schipper[64] has also reported that cottonwood tissues did not develop necrosis in response to mammatoxin.

Seedling explants of P. deltoides developed multiple adventitious buds analogous to P. tremuloides on shoot differentiation medium, and morphogenesis was unaffected by the presence of mammatoxin (Tables 8 and 9) even at levels that caused 100% mortality to organ cultures of P. tremuloides (Table 8). In addition, it was confirmed that cottonwood seedlings fail to develop necrosis in response to the fungal filtrate, as measured by the leaf puncture bioassay. The pooling of the leaf puncture bioassay results for 10 cottonwood seedlings from XD-0-85-72 formed the basis by which in vivo resistance was measured on seedlings and tissue culture propagules of quaking aspen (see Table 10). Thus, by one-way analysis of variance and Duncan's Multiple Range Test, toxin resistance in aspen was assigned based on statistically significant ($p = 0.05$) lesion diameters equivalent to those produced in cottonwood (0.91 ± 0.42 and 0.64 ± 0.53 mm for the H^+ EtOAc and H^+ n-BuOH extracts, respectively).

Table 8. Effect of mammatoxin on in vitro shoot proliferation of two Populus species[w].

Seedlot	Toxin, %	Shoot Induction Frequency, % Cotyledon	Hypocotyl
XT-3-83	0	60[a]	97[a]
	2	15[bc]	21[b]
	4	3[c]	6[b]
	8	0[c]	0[b]
XD-0-85-72	0	35[ab]	85[a]
	2	43[ab]	95[a]
	4	23[bc]	77[a]
	8	30[abc]	67[a]

[a,b,c]Within a column, means followed by a common superscript are not significantly different by one-way ANOVA with Duncan's Multiple Range Test ($p = 0.05$).
[w]Eight seedlings/plate; 4 plates/treatment; crude filtrate (257-6-41).

Table 9. Effect of mammatoxin on shoot production and callus growth for several seedlots[W].

Seedlot	Toxin, %	Callus[X], %	Fresh Weight, mg	Shoot Induction Frequency, %
XT-3-84	0	98[a]	24[a]	65[a]
	1	92[ab]	2.4[b]	8.3[de]
	2	83[bc]	2.5[b]	12[de]
	4	73[de]	1.7[b]	18[ce]
	6	68[ef]	2.3[b]	8.3[de]
	8	55[e]	--	0[e]
XT-2-84	0	98[a]	22[a]	50[ab]
	1	98[a]	6[b]	10[e]
	2	92[ab]	5.5[b]	0[e]
	4	82[bcd]	8[b]	0[e]
	6	75[cde]	2.8[b]	0[e]
	8	57[e]	3.1[b]	0[e]
XD-0-85-72	0	100[a]	--	43[abc]
	1	95[a]	--	48[ab]
	2	100[a]	--	31[bcd]
	4	85[bc]	--	22[cde]

[a,b,c,d,e]Within columns, means followed by common superscripts are not significantly different by one-way ANOVA with Duncan's Multiple Range Test ($p = 0.05$).

[W]Cotyledonary explants, 20 per plate, 3 plates/treatment, extracted filtrate (257-6-41).

[X]Designates the percentage of cultures that responded by callus formation, adventitious bud production, or a generalized swelling.

Table 10. Response to mammatoxin as measured by the leaf puncture bioassay of propagules derived from *in vitro* screening with crude fungal filtrates.

Seedlot	Explant	Clone	Ramet No.	Toxin Level, %	Lesion Diameter, mm[a] H^+ EtOAc	H^+ n-BuOH
XT-0-24-80	Hypo	STAR	6	10	0.67 (0.85)[b]	0.83
XT-3-83	Hypo	10A	7	2	0.86	0.89
		B7	6	2	0.84	0.84
	Cot	C6	6	2	0.95	0.90
	Hypo	A	2	4	0.78	0.92
		B	2	4	0.83	1.0
XD-0-85-72	--	--	10[c]	--	0.91	0.64

[a]For the lesion diameters obtained by testing with the extracts, all means within and between columns are not significantly different as determined by one-way ANOVA with Duncan's Multiple Range Test ($p = 0.05$).
[b]Mean lesion diameter for bioassay with crude filtrate from 257-6-10.
[c]Pooled bioassay of ten 18-week-old cottonwood seedlings.

Cottonwood was not without its difficulties as a control. As noted previously, adventitious buds produced could not be elongated by the micropropagation protocol. In addition, seedling explants of cottonwood did not respond with extensive callus proliferation as did approximately half the *P. tremuloides* cultures. As a consequence organogenically incompetent cottonwood cultures merely swelled and produced a minimal amount of callus, which made assessment of the effect of mammatoxin on callus production difficult.

When *H. mammatum* filtrates were extracted by the procedure of Griffin[100], toxic activity could only be detected in the acidic ethyl acetate and acidic n-butanol extracts (see Table 11). Because traces of n-butanol can be difficult to remove, 100 and 10% n-butanol-saturated water were also assayed. Saturation

Table 11. Response of selected seedlings to extracted mammatoxin (257-6-41) substances in the leaf puncture bioassay[a].

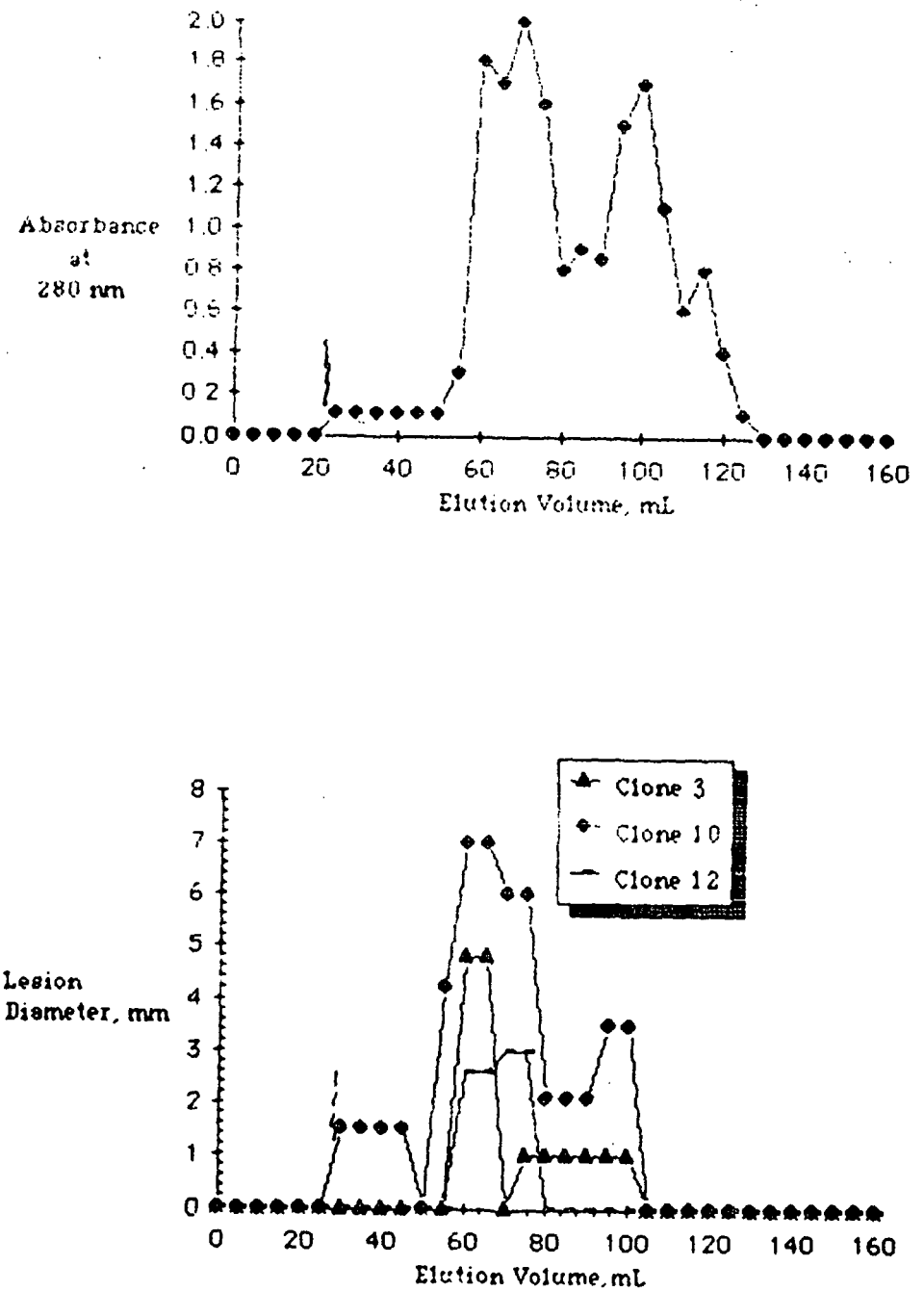
Seedlot	Seedling No.	Lesion Diameter, mm							
		Crude Filtrate	OH^- EtOAc	H^+ EtOAc	H^+ n-BuOH	Aqueous Remainder	Distilled Water	Water-saturated N-butanol	1/10 Water-saturated N-butanol
XT-3-83	2	7.2	0.61	6.8	6.1	1.0	0.64	7.0	1.0
	4	6.4	0.72	5.9	6.2	1.1	0.61	7.4	0.89
	6	4.4	0.56	4.8	4.0	0.89	0.72	8.5	0.93
XT-0-24-80	03	8.8	0.89	7.3	8.0	1.5	0.78	7.0	0.89
	04	5.3	0.93	4.9	5.0	0.93	0.93	9.5	0.77
	05	2.5	0.78	2.5	2.5	0.89	0.94	9.0	0.50

[a]Based on 2 punctures holes/leaf; 2 leaves/plant.

of n-butanol in water is 9% by weight, so that failure of a 10% saturated solution to cause necrosis means that for a typical extraction procedure, where the residue from the organic phase is taken up in 60 mL of water, 540 mg of n-butanol could be tolerated in the bioassay. However, the mass of the residue from evaporation of the n-butanol extracted never exceeded 100 mg. Note that even with water, some necrosis always develops in response to the puncture wound.

Griffin has previously shown that the distribution of mammatoxin between all the phases (organic and aqueous) is pathovar dependent[100]. However, regardless of the race of H. mammatum being cultured, some toxic activity was always associated with the organic extracts of the acidified filtrate, supporting previous observations that the toxin has the characteristics of an organic acid[64]. In isolate 257-6-41, this was strictly the case. In addition, it should be noted, because of the saturation of the response to each concentrated extract, the sum of the lesion diameter for each extract is not equal to the lesion diameter obtained for the unextracted filtrate, suggesting each preparation contains a toxin capable of inducing necrosis independently of other toxic agents.

Attempts to fractionate the extracts by gel-filtration chromatography were undertaken to further examine the nature of each extract (see Fig. 7 and 8). Not unexpectedly, the more polar n-butanol extracted the higher molecular weight fraction as indicated by the smaller volumes required to elute toxic activity. However, it should be noted that there is considerable overlap between the two extracts corresponding to the elution volume of the most toxic principle of each extract. Given that for sensitive clones, very low levels of a given extract will still produce a saturating response, it cannot be discounted that the extraction procedure was not efficient in separation of all the toxic principles,

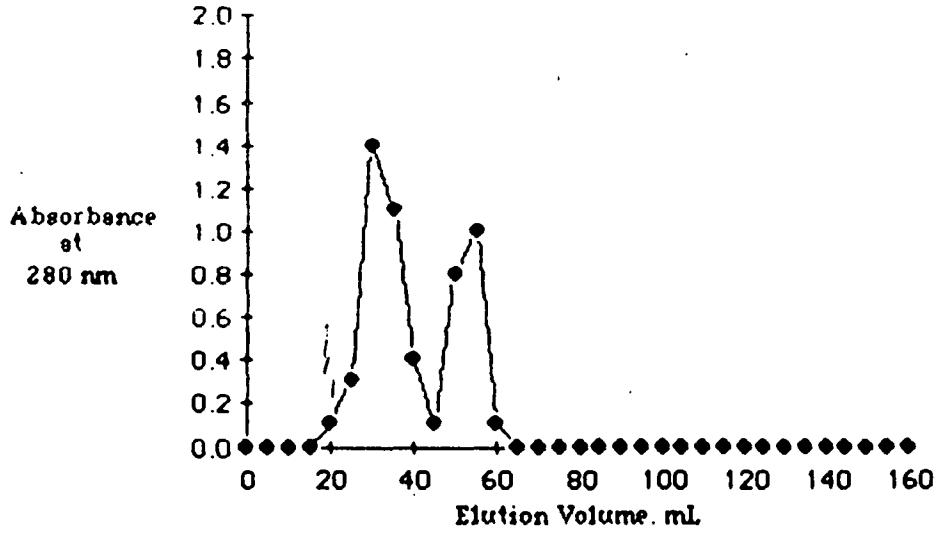


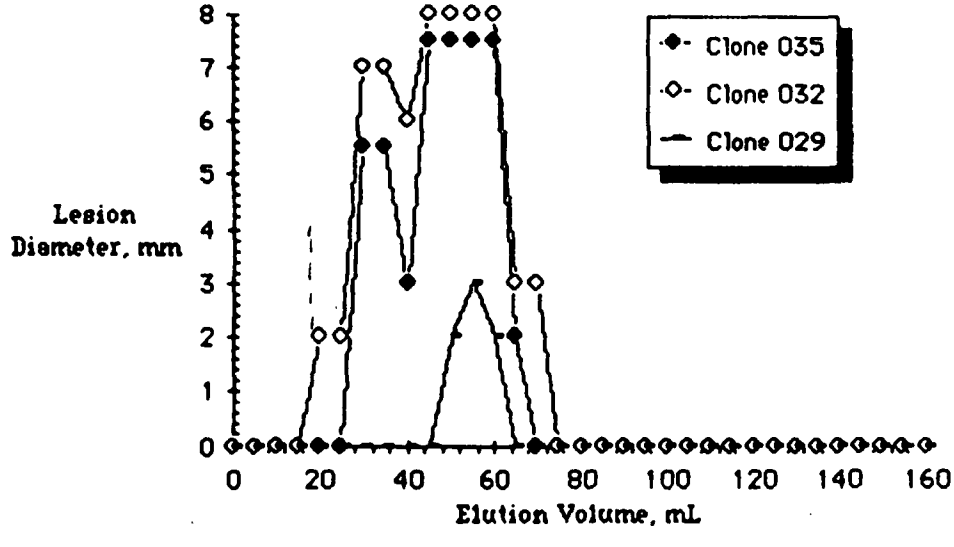
Fractions employed for further testing

fraction 5 (60-67 mL)

fraction 6 (67-78 mL)

Figure 7. Absorbance and lesion diameter vs. elution volume in gel-filtration chromatography of Hypoxylon mammatum cultural extracts (XT-3-83; H^+ EtOAc).





Fractions employed for further testing

fraction 5 (41-44 mL)	fraction 7 (48-54 mL)
fraction 6 (44-48 mL)	fraction 8 (54-60 mL)

Figure 8. Absorbance and lesion diameter vs. elution volume in gel-filtration chromatography of Hypoxylon mammatum cultural extracts (XT-0-24-80; H^+ n-BuOH).

such that each fraction may represent mainly quantitative rather than qualitative differences in toxic agents. This in turn may account for lack of statistically significant differences in lesion diameter elicited by each extract for a given clone.

Whatever the nature of the differences between extracts, they were sufficient to warrant a cursory examination of the response to fractionated toxin (Results Section; Bioassay of Tissue Culture Propagules and Seedlings), and in all other bioassays, the H^+ EtOAc and H^+ n-BuOH extracts were tested separately.

The whole leaf bioassay was initially employed, but was quickly abandoned because of its requirement for large amounts of toxin and, more importantly, it became inaccurate for young leaves (see Table 12). The whole leaf bioassay was first employed on seedlings from XT-0-24-80 and XT-3-83 as they entered the fall (see Appendix, Fig. 20). As the trees discontinued growth and began to set buds, the leaves reached the same state of development (i.e., fully expanded, flattened petioles, rigidity). Utilization of the whole leaf bioassay on this material produced necrotic areas that varied little between leaves and correlated well with the leaf puncture bioassay. However, the vast majority of other trees were not in a dormant state and it was undesirable to assay them due to questions concerning the effect of dormancy in the infection process. When actively growing trees were assayed, in which a spectrum of leaf maturity from base to crown was confronted, the whole leaf bioassay also began to show a spectrum of responses such that it became possible to observe 100 and 0% necrotic areas on leaves taken from the same plant! On the other hand, the leaf puncture bioassay was unaffected by leaf age, although leaves not fully expanded tended to be more sensitive to the toxin. Because "immature" leaves seemed to provide a more stringent test of toxin sensitivity, one less-than-fully expanded leaf was

Table 12. Whole leaf vs. leaf puncture bioassay.

1. Fully expanded leaves (XT-3-83)

Clone		Bioassay Results	
Seedling	Propagule	Whole Leaf, % necrosis	Leaf Puncture, mm[a]
18		100 ± 0	7.5 ± 1.2
19		4 ± 2	0.75 ± 0.22
20		0	0.83 ± 0.26
10		100 ± 0	9.0 ± 1.0
12		13 ± 8	2.8 ± 0.30
	T10	20 ± 11	2.5 ± 1.6
	T11	65 ± 20	2.4 ± 0.82
	T12	50 ± 28	2.4 ± 0.92
	T13	91 ± 12	3.7 ± 0.62
	T14	2 ± 1	0.67 ± 0.30
	T15	5 ± 5	3.9 ± 1.6
	T18	20 ± 10	2.3 ± 2.3
	T19	29 ± 9	2.5 ± 0.29
	T20	2 ± 0	0.72 ± 0.46

2. Immature leaves (XT-0-24-80)

T06	9 ± 4	4.5 ± 2.1
T029	6 ± 2	3.1 ± 1.7
T032	99 ± 2	2.2 ± 1.4
T020	62 ± 35	4.7 ± 2.7
T021	21 ± 21	2.2 ± 1.6
T022	64 ± 36	3.1 ± 2.2
T023	46 ± 36	2.1 ± 0.72
T024	61 ± 25	5.2 ± 1.3
T025	21 ± 13	4.4 ± 2.0
T026	20 ± 16	5.2 ± 1.8

[a]Value reflects average between the bioassay performed with H^+ EtOAc and H^+ n-BuOH.

always included in the triplicate assay of each plant. The imprecision of the whole leaf bioassay is reflected in the fact that all previous quantitative studies on mammatoxin employing leaf assays were performed by the leaf puncture method[71-75,100].

A situation that arises as a consequence of the micropropagation system is the question of "age" of the tissue culture trees. Because the tissue culture system produces a lag phase in height growth when compared to seedlings (Fig. 5), is it proper to assign an age based on when the cotyledon was explanted, or should age be equated to seedling height? The difficulty that may potentially arise is the validity in comparison of leaf puncture bioassays of trees of different ages. In actuality, this did not present a problem because the more pertinent question is not tree age but leaf age. Of the three leaves sampled per tree in the bioassay, all had the same relationship to each other in terms of age. The first leaf (immature) was the last less-than-fully expanded leaf (~ 3 weeks old). The next leaf was 5 cm lower on the stem (~ 5 weeks old) and was fully expanded, but still had a round petiole. The last leaf was 12-15 cm lower on the stem than the second (~ 9 weeks old) and was usually quite rigid as well as having a flattened petiole characteristic of leaves produced by mature trees. This same leaf age relationship (3, 5, 9 weeks) was employed in all plants tested in the leaf puncture bioassay, so the criterion of tree age was unimportant. However, with the exception of the bioassay of toxin-screened individuals from XT-0-24-80, where some plants were tested when they were 15 cm tall, all bioassays were not performed when the trees were 18 weeks old or reached a height of 75 cm, whichever came last.

In Vitro Experiments with Mammatoxin

In Vitro Isolation and Propagation of Mammatoxin Resistant Aspen

The application of various concentrations of crude and extracted filtrates from H. mammatum to aspen seedlings explanted on shoot proliferation medium resulted in a significant decrease in organogenesis (Tables 7-9). However, a small percentage of the cultures produced multiple adventitious buds in a manner indistinguishable from the cultures lacking toxin. Rescue and micropropagation of the survivors by the previously described procedure afforded plants that after approximately 26 weeks were challenged again with mammatoxin in a leaf puncture bioassay. In all cases (107 individuals representing 27 clones from four seedlots) these trees developed only a slight necrosis with no statistically significant difference from the response elicited by mammatoxin in cottonwood or by distilled water placed over the puncture holes of aspen.

Four experiments were performed using this method, which can be further divided into two categories. In the first category, the mammatoxin response of the donor plants subjected to in vitro screening was unknown, and in the second, the response of the donor plants was known. Discussion of the experiment representing the second category is deferred to a later section addressing the origin of the resistance response. The results of the three experiments representing the first category are discussed in the order in which they were performed.

The first experiment involved the screening of XT-0-24-80. The only screening of seedlings from XT-0-24-80 was with the isolate that was used only once (257-6-10). This open-pollinated seedlot was not screened again to avoid any possibility that a resistant, but interspecific hybrid might be obtained, and further screening with isolate 257-6-10 was discontinued because preliminary

bioassays suggested isolate 257-6-41 contained more toxin. However, this experiment is important to note because it was the only occasion on which tissue cultures were screened on one isolate and tested resistant to fungal filtrates from both isolates.

Rescue of the survivors from the 10% toxin substitution afforded only one single-explant clone (hypocotyl) that was ultimately established in soil (Table 7). Six tissue culture propagules (ramets) were established in soil and tested in the leaf puncture bioassay (6-32 weeks) with the crude filtrate from isolate 257-6-10 and the H^+ EtOAc and H^+ n-BuOH extracts from isolate 257-6-41. For both preparations, lesions failed to develop (Table 10).

Screening and biological assay employing multiple pathovars was beyond the scope of this work. The tacit assumption made was that different pathovars might produce different types and concentrations of mammatoxin, but that these toxins all share a common structural feature that imparts toxicity, and any differences in physical or chemical characteristics arise from minor structural modifications occurring in portions of the molecule unrelated to toxic activity. In the case of mammatoxin, there appears to be some precedent for this. French[71] found that different isolates produced different quantities of mammatoxin, but all toxic agents could be purified by the same sequence. Griffin demonstrated that different isolates produced toxins with different solvent partitioning capacities and gel-filtration elution profiles[100]. In addition, some isolates shared toxic principles as indicated by elution volumes. Again, all toxic principles could be separated by gels of similar exclusion volume (Bio-gel P-2 Sephadex LH-20, G-10). Both of these studies suggest, along with the early work of Schipper[64], that mammatoxin is a collection of very similar molecules that differ, at least in molecular weight.

In other pathogens employing toxin determinants of known structure this situation has been explored more intensively. The toxins produced by Helminthosporium Maydis (Race T) were found to be a series of related linear polyketols with 35-40 carbon atoms[65]. Also victorin (Helminthosporium sacchari) was found to contain a glycoside linked to a sesquiterpene that was isomeric with respect to the location of a double bond[65].

The hypothesis that screening with one pathovar imparts resistance to other races was demonstrated only in a preliminary manner in this work, not only by performing the bioassay with filtrates from different isolates, but throughout this work resistance to the H^+ EtOAc extract always meant resistance to the H^+ n-BuOH extract for isolate 257-6-41. Although these extracts seemed to have shared toxic activity, there nonetheless were still some qualitative differences. While further substantiation of this hypothesis would simplify practical efforts toward selection and breeding, it would also suggest a dangerous situation in terms of the immunity toxin resistance may impart. Briefly stated, if pathovars share a broad but similar range of toxins necessary for pathogenesis that can easily be selected for by employing a single, representative isolate, future pathogenicity may arise through major gene changes producing an entirely different toxin or some other altered mechanism of pathogenicity. The danger arises because the fungus could accomplish this many times more rapidly than the tree breeder could respond.

Because these were the first toxin-resistant plants produced by the in vitro screening system they received a considerable amount of attention. All six ramets were assayed repeatedly over a six-month period before they were planted in the IPC arboretum. All ramets exhibited resistance independent of the age of the tree. In one case, all the foliage of one of the ramets was

damaged by spider mites. This tree was treated with Pentac, cut back and still was resistant to mammatoxin when the new flush of growth was assayed. These observations nurtured the idea that toxin resistance was a stable characteristic (i.e., expression of resistance was not due to an epigenetic event) that was capable of being cloned, inasmuch as all six ramets demonstrated resistance to the same degree.

In the second screening experiment, XT-3-83 and XD-0-85-72 hypocotyls and cotyledons were placed on shoot proliferation medium containing 0, 2, 4, and 8% of the 2.5% concentrate of the crude filtrate from isolate 257-6-41 (Table 8). Recall that this represents 0, 4, 8, and 16% when comparison to screening with the extracted filtrate is made.

Regardless of the toxin level employed, the capacity to proliferate adventitious buds in surviving treatments was reduced to the same level for P. tremuloides and was unaffected for P. deltoides. For aspen, cotyledons and hypocotyls were suppressed to the same extent, but particularly noteworthy was the ability of cottonwood hypocotyls to proliferate shoots at a level equivalent to 16% substitution of the fungal filtrate. Clearly, over a considerable range of toxin concentrations, it appeared that the host-selective toxin was the active agent in the screening. However, it should be noted that elevated levels of mammatoxin caused 100% mortality to all aspen cultures. In this experiment, as in the first, explants either produced shoots analogous to the control cultures, or died within a week of initiation. The events leading to mortality of sensitive cultures were failure of the explants to swell, followed by a gradual browning, but not to the extent of blackening exhibited during mammatoxin bioassays. After turning color, the cells apparently lysed, such that observation after a week revealed only a "skin" of epidermal cells.

Rescue of the survivors, followed by micropropagation, afforded three and two clones from the 2 and 4% toxin treatment, respectively (Table 10). Just as organogenic frequency on toxin was unaffected by the concentration and explant type, resistance was expressed to the same degree in tissue culture propagules irrespective of explant or toxin concentration. The expression of resistance independent of explant type suggests that toxin screening is allowing expression of a trait independent of cell type; this is consistent with results obtained with host-selective toxins of other pathogens.

The final screening experiment in which the mammatoxin response of the donor was unknown was performed with reconstituted extracts (1:1, H^+ EtOAc:H^+ n-BuOH). Cotyledons from XT-3-84, XT-2-84, and XD-0-85-72 were explanted on shoot proliferation medium containing 0, 1, 2, 4, 6, and 8% levels of toxin substitution. A summary of the organogenesis and callus formation frequency is presented in Table 9, and a census of the type and number of resistant clones established in soil is presented in Table 13.

As before, the percentage of explants producing adventitious buds was independent of toxin concentration over a considerable range (1-6%), and resistant plants were regenerated in clones isolated from each level tested in XT-3-83. In XT-2-84, though a small percentage of buds were initiated on 1%, no organized development was observed at the higher levels of toxin. In addition, surviving cultures initiated on 1% toxin failed to elongate, so that no clones were established in soil as a result of in vitro screening of XT-2-84. It may be recalled from the results on the apparent genetic component to organogenesis that XT-2-84 responded poorly in vitro under toxinless conditions (Table 5).

Table 13. Census of mammatoxin-resistant plants regenerated during selection for toxin resistance[a].

Ortet Response Unknown			Resistant Ortets	
Toxin, %	Clone	Ramets, no.	Clone	Ramets, no.
1	R1-10	4	--	--
	R1-23	5	--	--
	R1-30	1	--	--
2	R2-13	2	2-18	1
	R2-14	2	2-26	1
	R2-15	8	2-34	2
	R2-34	4	2-60	1
	R2-42	2		
4	R4-7	1	4-22	1
	R4-10	1	4-34	4
	R4-19	5	4-37	2
	R4-21	2	4-38	8
	R4-28	1	4-47	2
	R4-35	3	20-24[b]	1
	R4-50	1	20-56[b]	1
	R4-56	4	20-74[b]	2
	R4-59	3		
6	R6-11	1	--	--
TOTALS:	18	50	12	26

[a]Cotyledonary explants screened on extracted filtrate. All clones derived from XT-3-84 unless otherwise noted.
[b]XT-20-84.

In contrast to screening with the crude filtrate, not all the cultures failing to undergo organogenesis died when the extracts were employed in vitro. Although a significant percentage did succumb to the toxin, approximately half the explants still survived toxin levels (as measured by callus formation) that failed to support bud differentiation. As in the study of the effect of polyamine synthesis inhibitors on aspen organogenesis[110], callus formation frequency included cultures undergoing organogenesis in addition to explants producing parenchymatous callus or a generalized swelling.

As measured by fresh weight, callus formation was significantly impaired, but it should be appreciated that an average mass of 2.5 mg represents over an eightfold increase in fresh weight from the initial cotyledon mass of 0.3 mg. In both XT-3-83 and XT-2-84, some of the larger calli were transferred to toxin-free shoot elongation medium but all died except for three calli from XT-2-84, which continued to grow and later differentiated roots on this medium. Except for these cases, the calli did not appear viable at the time they were examined, but it was apparent that they had survived at least a week on toxin-containing medium.

Despite the lack of knowledge of the mammatoxin response of the donor plants, some insight into the origin of the resistance response can be gained from these three screening experiments. Evidence that suggests this is not a somaclonal event are:

1. Stability of resistance response with respect to age and clone: Propagules tested resistant to the toxin at various ages up to 32 weeks. Shoot cultures were established in many of these clones, and as a result ramet production and subsequent assay were staggered over a six-month period. In all cases, shoot cultures continued to afford resistant plants. In each clone, all the ramets produced an identical response in the bioassay that was not significantly different in magnitude and standard error of the mean from cottonwood. Stability on this order is not always seen in somaclonal variants that can lose the tolerance they once expressed. In addition, culturally induced variation often does not exhibit the same degree of intraclonal uniformity.

2. Resistant plants were obtained regardless of the toxin level employed: Selection studies with cultured cells often show a dose-dependent response in cell growth parameters in the presence of stress agents. This dose dependency has been related to the somaclonal mechanism of gene amplification as a means of expression of stress tolerance. Dose dependency in stress tolerance has become so pervasive that it has been incorporated into protocols for selection studies. That multiple adventitious bud formation is independent of mammatoxin concentration over a considerable range, argues against a gene load mechanism for resistance.

3. Resistant plants were obtained regardless of explant type: Somaclonal variation has occasionally been associated with cultures derived only from certain explants. It has been forwarded that some organs may contain genetic

mosaics within somatic tissue that can be isolated and proliferated by a tissue culture system. The fact that toxin-resistant plants were obtained from both hypocotyls and cotyledons argue against this mechanism for the origin of the variation.

4. Selection for toxin resistance was infallible: For the 107 individuals propagated from toxin-containing media, the frequency of resistance in the propagules was 100%. The failure to observe "escapes" despite a brief selection interval is not characteristic of somaclonal variation, where several selection cycles are often required to reduce the frequency of escapes.

Germination of Seeds on Toxin-Containing Medium

Germination of seeds on agar-water medium that had been substituted with various levels of the crude cultural filtrate was envisioned as an alternative screening procedure. Indeed, the first successful report of screening for disease resistance employing a host-selective toxin was performed by a similar method. Luke and Wheeler[111] germinated over 100 bu (~ 4.5×10^7 seeds) of oats and soaked the young seedlings with a crude preparation of victorin, obtained from liquid cultures of Helminthosporium victorae. After four days, surviving seedlings were inoculated with the fungus, and 92% of the survivors demonstrated resistance.

Addition of the 2.5% concentrate of the crude filtrate (257-6-41) resulted in a marked reduction in germination that was seedlot dependent (Fig. 9). The dependence was apparently unrelated to the mammatoxin response because XT-3-83 was characterized by poor germination and reduced vigor of the resultant seedlings in the absence of the phytotoxin. In both seedlots, germination on toxin caused all the seedlings to appear damaged, and there was no observed differential in vigor.

When seedlings surviving the 8 and 4% toxin level in XT-0-24-80 and XT-3-83, respectively, were explanted onto shoot proliferation medium containing mammatoxin, all the cultures died. However, when these seedlings were explanted on

toxin-free medium, adventitious bud formation proceeded at or near the same frequency of explanted control seedlings that had been germinated on agar-water for seven days (see Table 14). As a result of the considerable level of shoot proliferation that occurred, clonal identity of the tissue culture propagules was not maintained in XT-0-24-80, but was, to an extent, in XT-3-83, where 16 individuals representing four clones were established in soil and assayed. In addition, in XT-3-83, 44 individuals of unknown clonal identity were also regenerated and assayed.

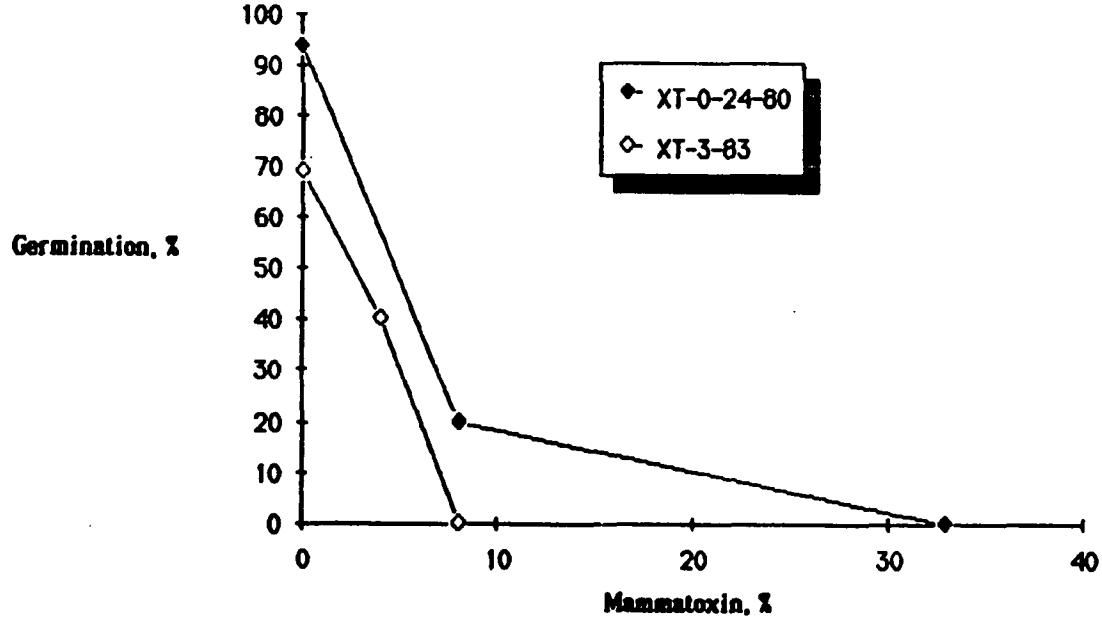


Figure 9. Effect of mammatoxin on seed germination.

The results of the bioassay on propagules derived from seed germinated on toxin is presented in Table 15. While the bioassay of seedling populations is discussed in the next section, it can easily be seen that 100% toxin resistance was not conferred to the propagules by this method with respect to the unscreened population (control). Due to the lack of clonal integrity in XT-0-24-80, it is impossible to comment on the efficiency of screening in this cross other than to

say it did not afford 100% resistance. In XT-3-83, the low number of clones examined make the uncertainty of the percentage of resistant clones shown in Table 15 on the order of 25%. As a consequence, it appears that the method applied to XT-3-83 is also inefficient. Lack of efficiency is also suggested in XT-3-83 by the comparable resistance (36 vs. 28%) obtained for a collection of individuals from an indeterminant number of clones.

Table 14. Organogenic behavior of toxin-germinated seedlings[w].

	Shoot Induction Frequency, %			
	XT-0-24-80		XT-3-83	
Toxin, %	Cotyledons	Hypocotyls	Cotyledons	Hypocotyls
0	77[a]	97[a]	60[a]	94[a]
0 (7-day control)	36[b]	52[b]	49[a]	82[a]
2	--	--	0[b]	0[b]
4	--	--	0[b]	0[b]
8	0[c]	0[c]	--	--

[a,b,c]Means in columns followed by a common superscript are not significantly different by one-way ANOVA with Duncan's Multiple Range Test ($p = 0.05$).
[w]Eight seedlings/plate, 4 plates/treatment; crude filtrate (257-6-41).

The reasons for the inefficiency or absence of selection in germinating on toxin-containing medium are not available from the results presented. Clearly, some manner of selection occurred, but apparently not for toxin resistance. An intriguing possibility is the inability of mammatoxin to be readily translocated. Schipper observed that large amounts of mammatoxin could be detected within the canker, but not in tissues 5 cm removed from the canker margin[64]. As noted previously, necrosis precedes the mycelial growth of the fungus, but does not produce long, necrotic streaks indicative of rapid transport of the toxin.

Evidence in support of this observation is that at a toxin concentration of 4% (crude filtrate), seedlings from XT-3-83 germinated at 58% of the control germination response (Fig. 9) while shoot proliferation only occurred at 5-6% of the control (4% extracted filtrate; Table 8). The ability of seeds from XT-0-24-80 to germinate on toxin levels lethal even to toxin-resistant cultures may also suggest selection is occurring by principles unrelated to the mammatoxin response.

Table 15. Mammatoxin response of propagules regenerated from toxin-germinated seedling (TG).

Seedlot	No. of Clones	No. of Individuals	Resistance, %[a]	
			Control	TG
XT-24-80	--	46	43[b]	17[c]
XT-3-83	4	16	28[b]	50[b]
	--	44	28[b]	36[c]

[a]Resistance is percentage of clones or individuals with a whole leaf bioassay < 10% (XT-0-24-80) or a leaf puncture bioassay not significantly different from cottonwood.
[b]Percentage of resistant clones in the population.
[c]Percentage of resistant individuals in the population.

Mammatoxin Bioassay of Seedling and Tissue Culture Propagule Populations

The results to this point indicate that mammatoxin resistance is a stable trait that can readily be isolated from the seedlots examined. The occurrence of selection is implied by the suppression of organogenesis on toxin containing medium, and the observation that toxin sensitive individuals occur in seedling and tissue culture trees propagated in the absence of toxin, but do not occur in populations regenerated from toxin-containing medium. The results of this section indicate the mammatoxin resistance or sensitivity is a naturally occurring trait, present in the seedlots examined and the propagules derived from these seedlings.

Figure 10 shows the result of the leaf puncture bioassay on six seedlots. In all cases, 25 seedlings were grown in the same soilless medium and assayed at the age of 18 weeks. In the seedlots examined, as indicated by the frequency of occurrence of lesion diameters between 0 and 1 mm, mammatoxin-resistant individuals were present to a greater-or-lesser degree depending on the parentage. Thus, it appears to a first approximation that somaclonal events that might lead to resistance expression are not required, and in this regard the tissue culture system merely serves as a convenient and efficient technique to rogue seedlots and subsequently propagate individuals with the desired resistance.

As noted previously, bioassays were performed keeping the H^+ EtOAc and H^+ n-BuOH extracts separate, despite the fact that there were rarely differences between the responses they elicited in a given tree. In Fig. 10 it will be noted that the frequency distributions depict a response to a single toxic principle. To derive a common lesion diameter for a tree tested with two separate extracts, advantage can be taken of the saturation of the toxin response at the assay concentrations to derive a "minimum lesion diameter" for each tree. This is readily accomplished by comparing the lesion diameter produced by each extract (H^+ EtOAc and H^+ n-BuOH) and reporting the larger of the two as the composite for the tree. In effect, this approximates the situation in which the two extracts were combined (1:1) and concentrated to half this volume. As noted, the lesion diameter that would be obtained is not equal to the sum of the fractions tested separately, nor would it be equal to the average response of the two fractions tested, but it would <u>at least</u> be the size of the largest lesion diameter obtained by testing each extract independently.

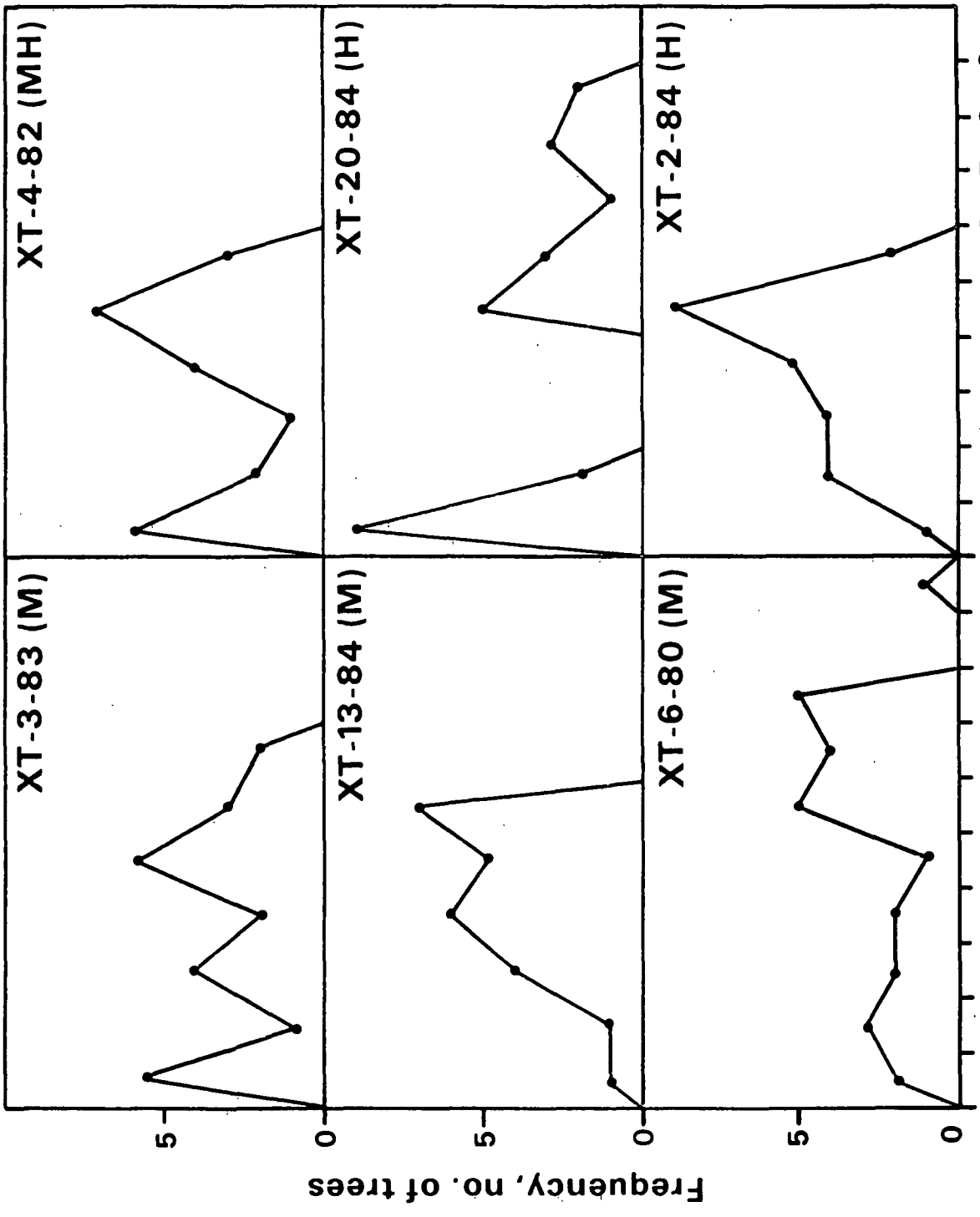


Figure 10. Frequency of mammatoxin response as a function of lesion diameter for six full-sib P. tremuloides crosses (25 seedlings/cross; value in parentheses indicates IPC Project 3250 hypoxylon rating system).

The data presented in Fig. 10 leaves the distinct impression that there exists a spectrum of mammatoxin responses in each seedlot. While analysis of variance performed on the 25 seedlings from each cross revealed significant differences between trees, the grouping of common means by the Duncan's Multiple Range Test revealed that, especially for toxin sensitive trees, very few groups are obtained; the bioassay is incapable to a large extent of detecting differences between the severity of the toxin reactions (Table 16; Appendix, Table 22).

A similar situation also arises in trees exhibiting low sensitivity to mammatoxin. In these cases, lesion diameters on the order of 2-3 mm are not significantly different from trees that are virtually unreactive toward mammatoxin (lesion diameters $\leq$ 1 mm). This situation can be rectified by comparing these means and their standard errors with the response exhibited by cottonwood. In the case of cottonwood (and aspen with mean lesion diameters $\leq$ 1 mm), the average lesion size reported for each extract is based on nine inoculations that in all cases failed to develop necrosis. The result is a small average lesion diameter with a small standard error corresponding to this average. Thus, the grouping of slightly reactive and unreactive trees by Duncan's Multiple Range tests is in a sense artificial because slightly sensitive trees can exhibit a substantial range of lesion diameters in each test spot, that only through averaging turn out to be small. This is a distinctly different response than that exhibited by resistant individuals, and it is reflected not in the average lesion diameter, but in the standard error of the mean. Thus, as noted before, resistance in aspen is only assigned on the criteria that no lesions are formed in all 18 test spots, resulting in a mean and its standard error equivalent to cottonwood (i.e., 0.91 $\pm$ 0.42 and 0.64 $\pm$ 0.53 mm for the H^+ EtOAc and H^+ n-BuOH extracts, respectively).

Due in part to the imprecision in the bioassay, the mammatoxin response of these seedlots might be best characterized as "on-off." Seedlings either develop no lesions in all leaf puncture holes inoculated as in cottonwood, or develop lesions of some average size showing very little difference in the extent of necrosis between trees.

Table 16. Mammatoxin response of two full-sib aspen crosses.

(Leaf Puncture Bioassay, 18 weeks)[w]

Tree No.	H^+ EtOAc Mean Lesion Diameter, mm	Tree No.	H^+ n-BuOH Mean Lesion Diameter, mm
	XT-3-83		
6	6.4 a	15	5.1 a
15	6.1 a b	6	4.8 a b
18	5.2 a b c	11	4.2 a b c
8	6.1 a b c	9	4.0 a b c
11	5.1 a b c	23	3.9 a b c
9	4.7 a b c d	3	3.6 b c d e
3	4.6 a b c d	1	3.4 c d e
17	4.6 a b c d	18	3.3 c d e f
23	4.6 a b c d	5	3.3 c d e f
13	4.4 a b c d	13	3.2 c d e f g
1	4.1 b c d e	8	2.6 d e f g
5	4.1 b c d e	10	2.4 e f g h
20	3.8 c d e	20	2.2 e f g h i
10	3.2 c d e	17	2.0 f g h i j
7	2.8 d e f	2	1.8 g h i j
24	2.4 e f g	24	1.4 g h i j
2	2.1 e f g	7	1.4 g h i j
19	2.1 e f g	16	1.3 g h i j
16	1.1 f g	19	1.3 g h i j
25	0.89 f g	25	0.94 h i j
14	0.72 f g	21	0.89 h i j
21	0.72 f g	22	0.78 i j
22	0.72 f g	4	0.72 i j
4	0.61 g	14	0.61 i j
12	0.61 g	12	0.56 j

See end of table for footnote.

Table 16 (Continued). Mammatoxin response of two full-sib aspen crosses.

(Leaf Puncture Bioassay, 18 weeks)[w]

Tree No.	H^+ EtOAc Mean Lesion Diameter, mm	Tree No.	H^+ n-BuOH Mean Lesion Diameter, mm
	XT-20-84		
20	8.3 a	20	7.3 a
18	8.0 a b	7	6.9 a b
13	7.7 a b	18	5.9 a b
3	7.2 a b c	13	5.9 a b
16	7.2 a b c	3	5.8 a b
7	6.9 a b c d	15	5.8 a b
15	5.8 b c d e	16	5.6 a b
24	5.2 c d e	11	5.1 b c
9	5.1 c d e	1	4.3 b c d
1	4.9 c d e	23	4.2 b c d
21	4.7 c d e	9	4.1 b c d
23	4.7 c d e	24	3.7 c d e
11	4.6 d e	21	3.2 d e
4	4.2 e	4	2.1 e f
14	1.5 f	14	1.3 f
8	1.0 f	8	0.89 f
6	0.89 f	19	0.89 f
10	0.89 f	6	0.83 f
12	0.89 f	17	0.83 f
19	0.83 f	10	0.78 f
17	0.78 f	12	0.78 f
25	0.78 f	2	0.72 f
2	0.72 f	25	0.72 f
22	0.72 f	5	0.67 f
5	0.56 f	22	0.61 f

[w]Within a column, means followed by common letter are not significantly different as determined by one-way ANOVA with Duncan's Multiple Range Test ($p = 0.05$).

Rather than express the mammatoxin response in Fig. 10 as simply the percentage of resistant individuals, a frequency distribution graph was used to illustrate qualitatively the two distinct profiles obtained in these seedlots. Despite the fact that these response profiles are somewhat artificial, it

appears seedlots exhibit a Gaussian distribution of toxin responses (XT-2-84, XT-13-84, and XT-6-80) or a distribution that is bimodal (XT-4-82, XT-3-83, and XT-20-84).

In a study noted earlier on resistance by callus formation after artificial inoculation with H. mammatum, Valentine noted a similar pattern in the distributions of callus formation among half-sib progeny groups[41]. He found that callus formation within these half-sib families sometimes occurred at discrete percentages but occasionally was continuous within a family. From the data presented here, it can be suggested that the distinct bimodality of some crosses could account for discrete groupings of callus formation as a resistance mechanism in vivo. For example, XT-13-84 and XT-20-84 are half siblings having a common mother, T-1-58. If the percentage of mammatoxin-resistant individuals in these crosses were equated to the ability to produce callus when artificially inoculated, it would occur at 8 and 44% for XT-13-84 and XT-20-84, respectively. Although there is no telling how a third half-sib cross with T-1-58 would behave, it does not seem unreasonable to suggest that bimodal distributions for toxin response could effectively translate into gaps in callus formation ability. The type of Mendelian segregation that occurs in callus formation is consistent with oligogenetic traits which are in turn characteristic of resistance to diseases employing toxin determinants. In addition, it will be recalled, mammatoxin has been demonstrated to inhibit callus formation in vitro[37,64].

As a final part of this section, consider the reaction to fractionated (gel filtration chromatography) mammatoxin of a population of tissue culture derived propagules from XT-0-24-80 and XT-3-83 (Fig. 11-17). The propagules from each seedlot were established in studies aimed at developing the micropropagation protocol and again, the clonal integrity was not maintained in either population.

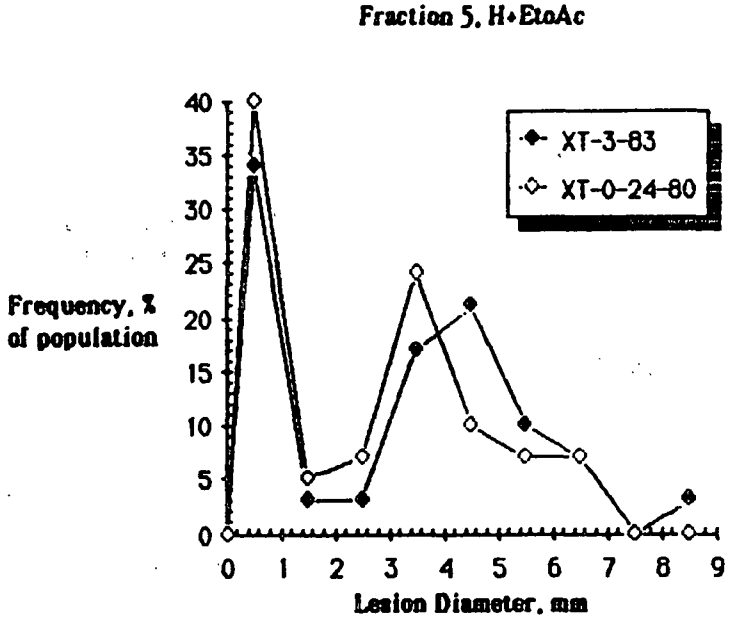


Figure 11. Effect of extract fractionation on leaf puncture bioassay of tissue culture propagules.

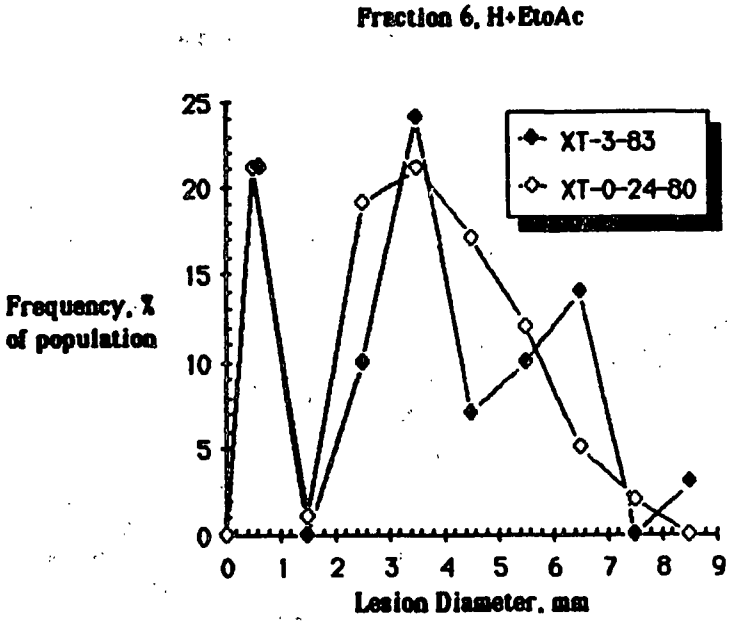


Figure 12. Effect of extract fractionation on leaf puncture bioassay of tissue culture propagules.

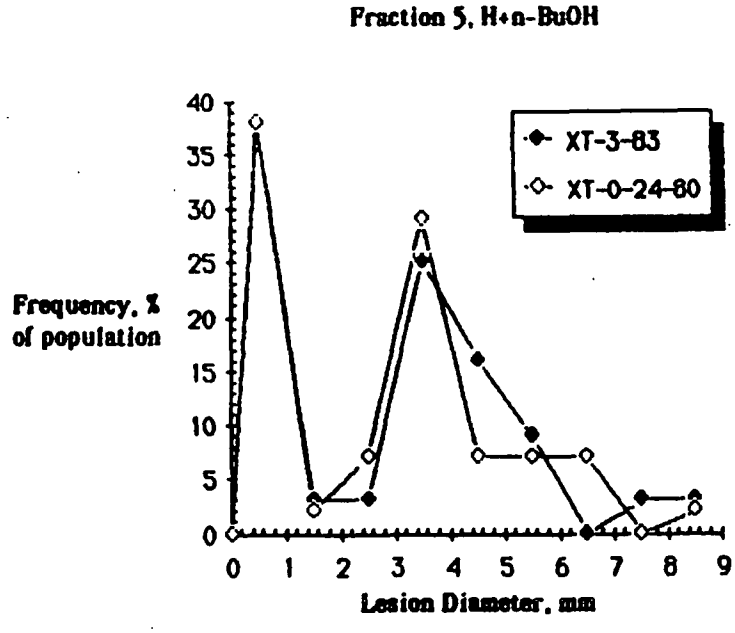


Figure 13. Effect of extract fractionation on leaf puncture bioassay of tissue culture propagules.

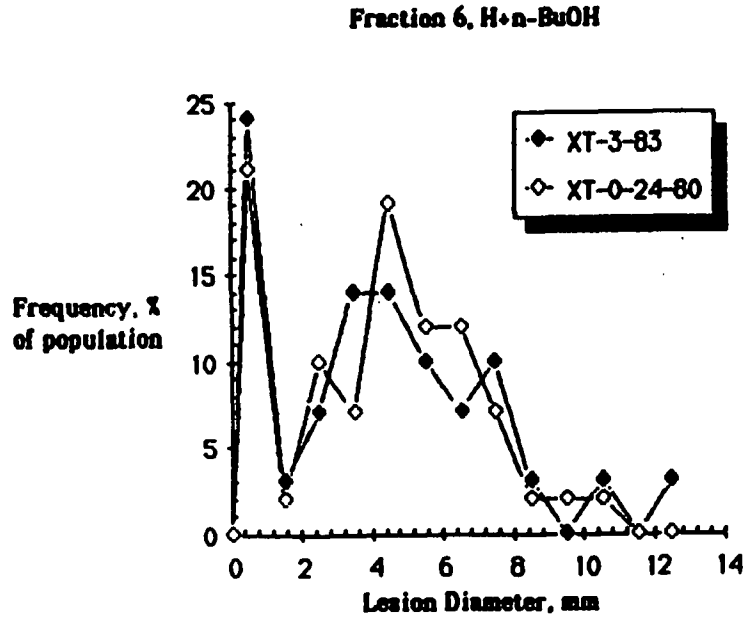


Figure 14. Effect of extract fractionation on leaf puncture bioassay of tissue culture propagules.

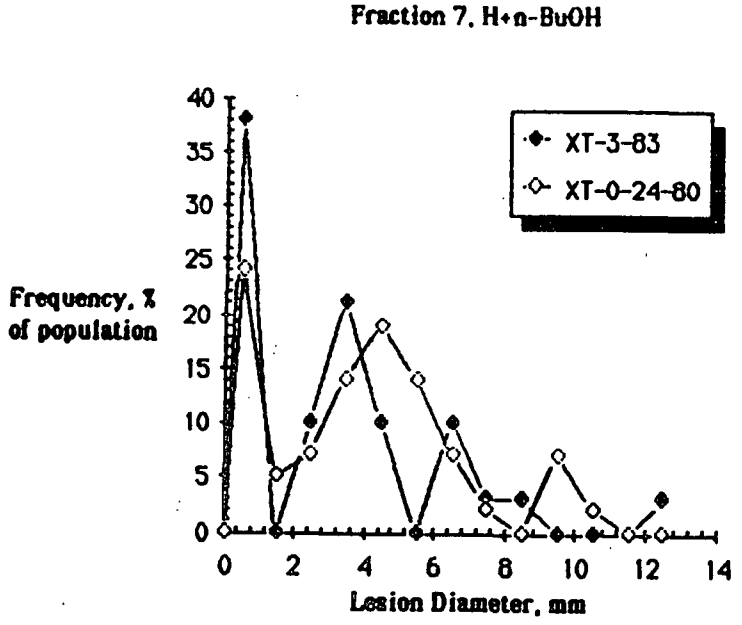


Figure 15. Effect of extract fractionation on leaf puncture bioassay of tissue culture propagules.

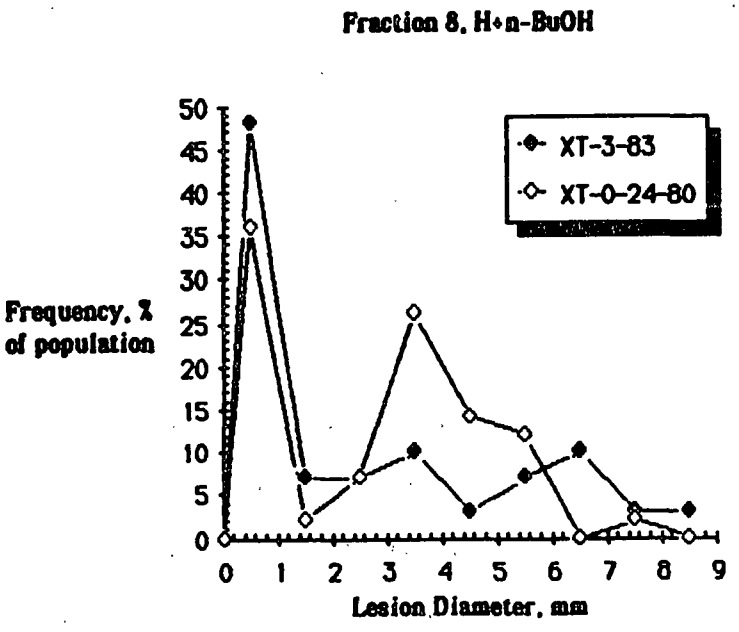


Figure 16. Effect of extract fractionation on leaf puncture bioassay of tissue culture propagules.

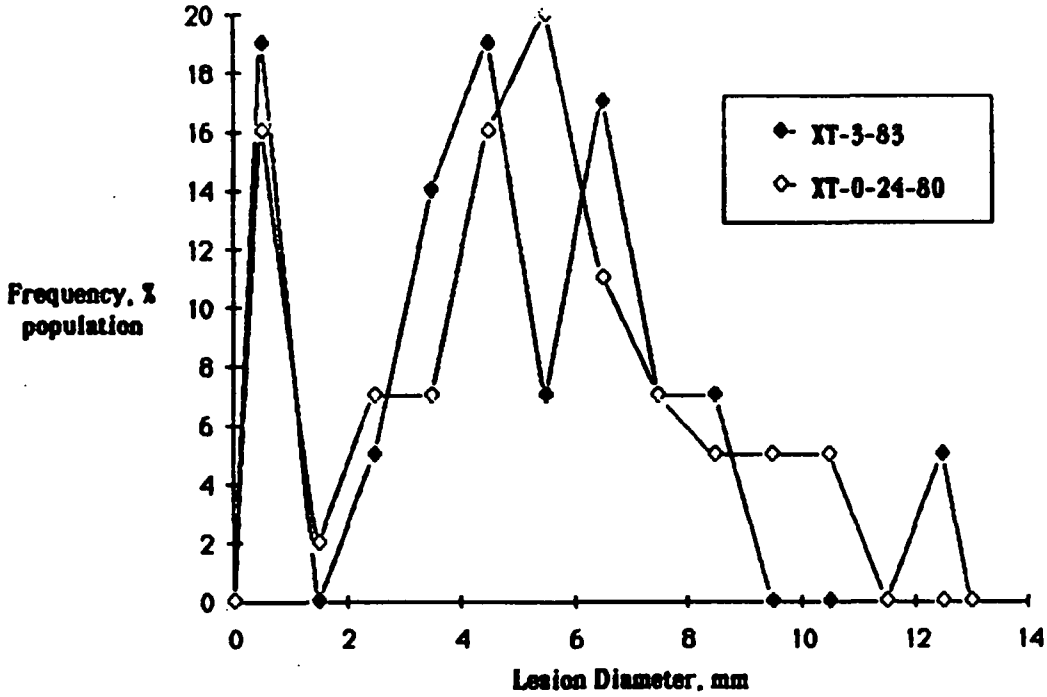


Figure 17. Composite mammatoxin response (Fig. 11-16) for the leaf puncture bioassay of tissue culture propagules.

However, during the development of the protocol, attempts were not made to intensively harvest explants producing a large number of shoots. Thus, while each of the 42 propagules assayed per seedlot do not represent an individual seedling (clone), the number of clones represented is likely to be large enough to reflect the distribution of the mammatoxin responses in each of these seedlots.

While it is difficult to separate qualitative from quantitative toxin differences between each of the six fractions tested in Fig. 11-16, some indications of differential sensitivity can be detected. In particular, fraction 6 of both the H^+ EtOAc and H^+ n-BuOH extracts appears considerably more toxic than fractions 5 and 8 of the H^+ EtOAc and H^+ n-BuOH extracts, respectively. In addition, for each fraction tested, a considerable percentage of the propagules

failed to form lesions, although it is not necessarily the same propagules failing to react in each fraction tested. Invoking the "minimum lesion diameter" concept again (Fig. 17) allows formulation of a composite distribution similar to those shown in Fig. 10. Comparison of the composite distribution of the tissue culture propagules to seedling populations in both crosses (Fig. 10, Fig. 20) revealed considerable similarities. In XT-3-83, the distribution of propagules and seedlings (as measured by the leaf puncture bioassay) are both bimodal, with maximums centered at a lesion diameter of 1 and 3-5 mm. In comparing the leaf puncture bioassay of the propagules to whole leaf bioassay of XT-0-24-80 and XT-3-83 some indication of the conversion factor to equate the bioassays is given for mature leaves in Table 12. By taking resistance as $\leq$ 10% necrotic area, the whole leaf bioassays of both seedlots are distinctly bimodal.

However, the similarity of the percentage of resistant individuals in the propagule population (19 and 16% for XT-3-83 and XT-0-24-80, respectively) with the percentage resistance in seedling populations (28%, XT-3-83; 44% XT-0-24-80) was not as evident. It should be noted that the lack of knowledge of clonal integrity of the propagules could be such that if one or several clones were represented by enough propagules it could skew the distribution from an accurate representation of the seedling population. Whatever the case, the important point is that regardless of absolute number of clones examined, a sufficient number were assayed to demonstrate that mammatoxin response is independent of the organogenetic competency of the genome in question.

Although this may appear to be a trivial conclusion for the preceding convoluted argument it bears further consideration. There is no reason *a priori* to expect the phytotoxin response to be linked to the ability to produce multiple adventitious buds *in vitro* or any other desired trait. General acceptance of

this notion is evident in the choice of cultivars with high morphogenic potential in selection studies in agronomic crops[87]. In this study, the lack of correlation between the mammatoxin response and any trait of value to the pulp and paper industry highlights a problem with a selection system based on seedlings. In particular, there is no reason to expect the mammatoxin response to be linked to growth rate, form or specific gravity. The latter traits take many years to evaluate and cannot be ascertained from examination of seedlings. Thus, even if in vitro screening of seedlings with mammatoxin could afford hypoxylon canker resistance, and the concomitant increase in volume growth through reduced mortality from the disease, unintentional, coincidental selection for reduced growth rate could conceivably result in no net gain in volume growth. To gain an appreciation of the number of seedlings that potentially would have to be screened to achieve a successful combination of growth rate and form with mammatoxin resistance, consider that in the normal, diploid crosses examined here, the frequency of outstanding individuals is around 4%[112]. In XT-3-83, where toxin resistance occurs at 28%, on the order of 100 seedlings (0.04 x 0.28) would have to be screened to obtain one clone exhibiting both traits. Fortunately, this screening could be accomplished in a couple of hours in five 9-cm diameter Petri dishes. However, further confirmation of the successful combination of these traits would still require years to resolve.

In summary, it has been shown that resistance and susceptibility to the necrotic effect of mammatoxin is present in seedling populations. Plants regenerated from culture of these populations reflect a mammatoxin response similar to the donor population, suggesting the mammatoxin response is cloned and expressed independent of in vitro morphogenic potential. Further evidence for clonal propagation comes from the observation that in all resistant single-explant

clones established, all the ramets within a clone exhibited the identical, resistant response.

It seems apparent that the screening method employed only allows organogenesis to proceed in the resistant individuals already present in the population. This resistance is subsequently expressed in all regenerated propagules due to the clonal nature of the micropropagation protocol. Confirmation of this hypothesis can only be made by comparison of the mammatoxin response of a plant regenerated from a toxin-resistant culture to the response of the plant that the culture was initiated from.

Mammatoxin Response of Propagules Regenerated from Cultures of Known Response

The mammatoxin response of tissue culture plants regenerated from seedlings of known response was obtained by explanting a single cotyledon from donor plants and growing the remainder of the seedling. This was performed in the presence and absence of mammatoxin, forming the basis for two experiments. In the first experiment, to investigate the clonal nature of the propagation system with respect to the mammatoxin response, 24 single cotyledon cultures (XT-3-83) were employed to regenerate six tissue culture ramets per clone. Comparison of the mammatoxin response of these ramets to the seedling (ortet) from which they were derived formed the basis for 24 seven-member families (Ortet-Ramet Bioassay).

The second, critical experiment was performed in a similar manner, except that single cotyledon explants (XT-3-84, XT-20-84) were explanted onto shoot proliferation medium containing mammatoxin. In all cases, seedlings corresponding to organogenic cultures were assayed, but not all the cultures producing multiple adventitious buds could be successfuly established in soil (Table 17). Although the mammatoxin response of several clones regenerated from

toxin-resistant cultures was compared to the donor plants, this experiment is perhaps best considered as an "in vitro bioassay."

Table 17. Effect of mammatoxin on shoot production in Ortet-Ramet families[w].

Seedlot	Toxin, %	Shoot Induction Frequency, %	Ortet-Ramet Families	
			Observed[x]	Established[y]
XT-3-83	0	63[a]	48	24
	2	18[bc]	11	4
	4	13[bc]	5	5
XT-20-84	0	53[ab]	---	---
	4	4[c]	4	3

[a,b]Within the column, means followed by a common superscript are not significantly different by one-way ANOVA with Duncan's Multiple Range Test (p = 0.05).
[w]Cotyledonary explants, 20 per plate, 3 plates/treatment; extracted filtrate (257-6-41).
[x]Mammatoxin response of ortet corresponding to organogenic cotyledon known.
[y]Mammatoxin response of ortet and tissue culture propagule known.

Ortet-Ramet Families

From 120 cotyledons placed on shoot proliferation medium 48 shoot cultures were established. From these shoot cultures, 24 were chosen on the criteria of survival of the donor seedling and in vitro proliferative potential (Table 17).

The 24 families (1 ortet + 6 tissue culture ramets) were subjected to linear regression analysis by comparison of the average lesion diameter of the ortet to average lesion diameter for the six ramets, and the results are shown in Fig. 18.

To reiterate, the first observation to be noted in Fig. 18 is the independence of mammatoxin response and organogenic capability. The second feature to

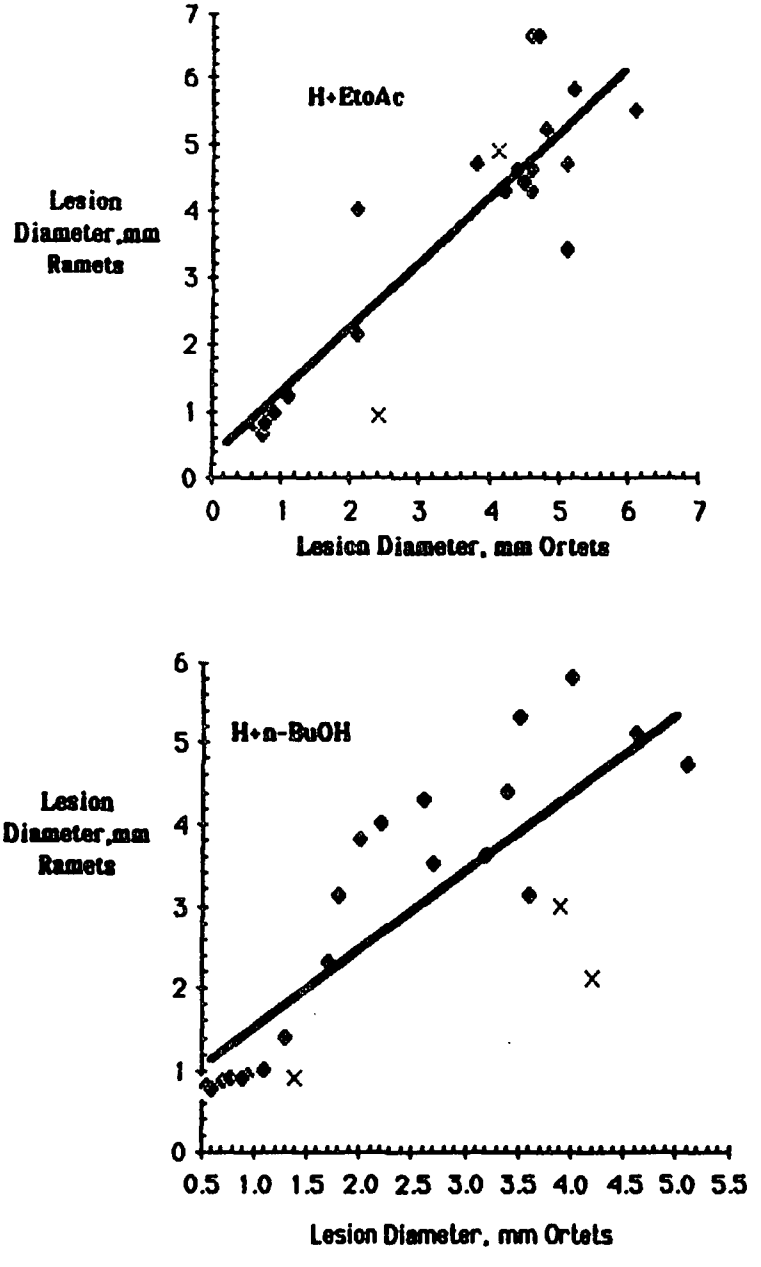


Figure 18. Equivalency of mammatoxin response of 24 seedlings (ortets) and their corresponding tissue culture propagules (ramets) as measured by the leaf puncture bioassay. Ramet response represents the average value of 6 propagule/ortet (ortet/ramet families exhibiting statistically significant variation in lesion diameter are marked with a "x").

note is the high degree of correlation (H^+ EtOAc, $r^2 = 0.91$; H^+ n-BuOH, $r^2 = 0.81$), which for the 22 degrees of freedom represented by these 24 paired values is significant at greater than the 99% confidence level.

However, it should be noted that the average value in no way relates to how the bioassay of the individual ramets compares to the ortet. To accomplish this, one-way ANOVA ($p = 0.05$) was performed on each ortet-ramet family, and families containing statistically significant differences in lesion diameters for a given extract are marked with an "x" in Fig. 18. For these families, Duncan's Multiple Range Test ($p = 0.05$) was performed to rank the means within a family. Of the 48 familial relationships, significant differences were obtained in five clone-extract families - two in the assay with H^+ EtOAc and three in the assay with H^+ n-BuOH. The leaf puncture bioassay for the ortets and ramets of these variant families are presented in Table 18. Probably the most intriguing aspect of these variants is that in four of the five cases, the ramets exhibited significantly smaller lesion diameters than their corresponding ortets. This observation is surprising because even though the slope of the regression line is nearly unity, both extracts have a nonzero intercept indicative of the general trend for the ramets of a given clone to produce slightly larger lesions than the ortet.

While it may be tempting to cite this as evidence for somaclonal variation, several observations on the bioassay suggest caution in making this statement. Griffin[100] has observed day-to-day variations in the bioassay response, and the lack of precision in the assay as it was employed in this work, both argue for a source of variation introduced by the experimental procedure. Bruck and Manion[74] have also demonstrated that the lesion diameter obtained in the bioassay can be

Table 18. Variation in clonal response to mammatoxin (XT-3-83)[w].

Clone	Extract	Lesion Diameter, mm	
		Ortet	Ramet
1	H^+ EtOAc	4.1^{ab}	5.9^{a}
			5.8^{a}
			5.8^{a}
			5.4^{a}
			4.2^{ab}
			3.2^{b}
73	H^+ n-BuOH	4.2^{a}	3.9^{a}
			2.8^{ab}
			2.7^{abc}
			1.3^{bc}
			1.1^{bc}
			1.0^{c}
96	H^+ n-BuOH	3.9^{b}	6.1^{a}
			5.8^{a}
			5.7^{a}
			1.7^{c}
			1.9^{c}
			1.6^{c}
111	H^+ EtOAc	2.4^{a}	1.1^{b}
			1.1^{b}
			0.89^{b}
			0.89^{b}
			0.89^{b}
			0.61^{b}
111	H^+ n-BuOH	1.4^{a}	1.1^{ab}
			0.94^{b}
			0.89^{b}
			0.89^{b}
			0.83^{b}
			0.78^{b}

[a,b,c]Within each entry, means followed by common superscript are not significantly different by one-way ANOVA with Duncan's Multiple Range Test ($p = 0.05$).

[w]Cotyledon explants, propagated under toxin-free conditions.

influenced by the nutrient status of the tree. This may be particularly important when considering the bioassay results of tissue culture trees. Many of the clones that comprised the ortet-ramet bioassay were propagated only for the evaluation of the mammatoxin response and then disposed of. In the process of throwing them away, the root systems were always examined and in many instances, the mass of roots appeared substantially less than that for seedlings. However, this did not result in reduction of height growth, nor were variant bioassay results associated with plants exhibiting attenuated root growth. However, it may not be unreasonable to suggest unobservable factors affecting nutrient status of the plant could have been produced as a consequence of *in vitro* manipulation and resulted in aberrant bioassay results. Thus, while somaclonal variation is still a possibility, a more detailed analysis (especially elemental analysis) than just the mammatoxin bioassay would have to be performed on the variant families. In this regard, it suffices to say that ramets always appeared indistinguishable from the ortet in terms of leaf shape, number of serrations, and in some cases, even such quirks as a tendency toward epinasty.

The *In Vitro* Mammatoxin Bioassay

Comparison of the mammatoxin response of propagules regenerated from toxin-resistant cultures to their corresponding ortet revealed no variation in response in the ortet-ramet families as noted above. All propagules regenerated from toxin-containing medium tested resistant to mammatoxin, and corresponded to an ortet that also was resistant. The collection of these resistant ortet-ramet families is depicted in Table 13. Once again, resistance was obtained in XT-3-84 regardless of the level of toxin employed, and resistant clones were established from XT-20-84, a progeny group characterized by a very high incidence of hypoxylon canker. Isolation of resistant individuals from progeny groups that have

T-1-58 as the female parent is particularly noteworthy because these groups usually exhibit outstanding form and volume growth, but are also characterized by a high incidence of hypoxylon canker.

This procedure has been termed an in vitro bioassay because all of the cultures producing multiple buds could not be established in soil, but the response of the ortet corresponding to these cultures could be determined. Segregation of these ortets from the rest of the population resulted in a new, second-order population of seedlings having the common ability to undergo organogenesis when explanted on toxin-containing medium. Comparison of the mammatoxin response of the second-order seedling population with the original population is shown in Fig. 19. In both XT-3-84 and XT-20-84 a high level of resistance was obtained. In XT-30-84, 100% resistance was exhibited by the second-order population, but this was based on a population of five seedlings, so the uncertainty is on the order of 20%. In XT-3-84, 16 seedlings comprised the population, and 96% resistance was obtained. However, one seedling did produce small lesions (~ 1.5 mm for both extracts) suggesting complete toxin resistance may not be an absolute requirement for regeneration on toxin containing medium. However, while buds were produced, no shoots were ultimately elongated or established in soil from the culture corresponding to this seedling.

The infallibility of shoot proliferation on toxin-containing medium as a criterion for resistance implies that an in vitro bioassay employing this criterion could result in:

1. Replacement of the leaf puncture bioassay - In the first bioassay of seedling populations (XT-0-24-80 and XT-3-83; whole leaf bioassay, see Appendix Fig. 20), 65 seedlings from each cross were examined. When it was discovered

that the distribution of mammatoxin response based on 20 seedlings was the same as for 65, further assays with the leaf puncture method in other seedlots were performed on 25 individuals, as this number of seedlings seemed to give a fair representation of the seedlot. To grow 25 seedlings for 18 weeks required about 9 cubic feet of greenhouse space in addition to daily attendance to care for the seedlings. The in vitro bioassay on the other hand required negligible space (hundreths of a cubic foot), less time (four weeks) and only several hours of effort to aseptically germinate and explant seedlings. The method may have limitations in organogenically deficient crosses, but because there is no requirement that the in vitro bioassay needs to result in steckling formation, the limitations may be minimal as in the case of XT-2-84.

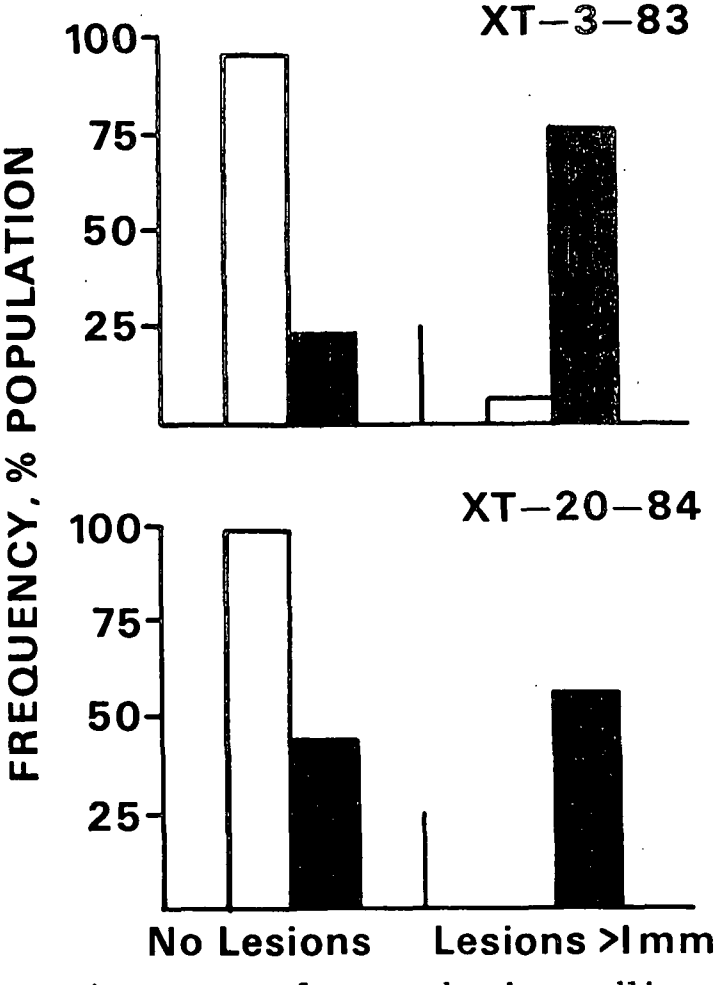


Figure 19. Mammatoxin response of a second-order seedling population derived by the in vitro bioassay (open bars = second-order population; closed bars = control population).

2. Establishment of second-order progeny groups - Employing the single-cotyledon in vitro bioassay, seedlings corresponding to proliferating cultures could be identified and lined out in a nursery similar to the method used to produce acceptable aspen planting stock from seed. The best estimate for the rate at which this could be performed (including media preparation, aseptic germination, dissection and plating of cotyledons, and transfer of single-cotyledon seedlings to soil) is one minute/seedling. The level of skill required to perform this task is not particularly high, (as witnessed by the fact that I could do it) such that for reasonable labor costs, thousands of seedlings could be evaluated and established in a week.

The appeal of both these methods is the lack of a requirement for the in vitro system to produce plants. Although toxin-resistant shoot cultures could be established and produced a continuous source of rootable shoots, the micropropagation system established here is a long way from being a cost-effective method of plant production. Furthermore, the long-range performance of tissue culture propagules in woody species is still an unanswered question. Given this current state of affairs, initial application of this method may be best suited as a tool to augment and be integrated into an existing aspen improvement program.

CONCLUSIONS

The addition of filtrate preparations from liquid-grown cultures of Hypoxylon mammatum to seedling explants of Populus tremuloides cultured on a medium formulated to produce multiple adventitious buds resulted in a marked reduction in the frequency of organogenesis. Rescue and subsequent micropropagation (by a protocol developed in this work) of the survivors afforded plants that when challenged with the filtrate preparations in a leaf puncture bioassay did not respond with the typical necrosis characteristic of aspen. Instead, propagules derived from this procedure developed only a slight necrosis associated with a wound produced in bioassay. This response was identical to that produced in Populus deltoides, a related species not susceptible to hypoxylon canker or the necrotic effects of the filtrate. The observation that the filtrate preparations did not affect P. deltoides in vivo or in vitro supported the supposition that mammatoxin was the active principle of these preparations.

By performing the leaf puncture bioassay on seedlings grown from the same seedlots that were screened in vitro, the source of the resistance response was traced to the natural variation in mammatoxin response already present in these full-sib progeny groups to a greater-or-lesser degree.

The general acceptance of the notion that micropropagation schemes similar to the one developed here constitute a clonal method of plant propagation implies that the application of mammatoxin to in vitro culture allows only those genomes endowed with resistance to selectively proliferate. This notion was tested in both the absence and presence of mammatoxin by establishing tissue cultures from single cotyledons and growing the remainder of the seedling. In this way, the mammatoxin response of the tissue culture propagules (ramets) could be directly compared to the seedling (ortet) from which they were derived.

When the mammatoxin response of 24 ortet-ramet families produced by organogenesis on toxin free media was evaluated for two filtrate extracts, the response of the ramets was usually indistinguishable from that of the ortets, suggesting that this micropropagation scheme is clonal. In addition, observation that toxin-resistant and susceptible ortet-ramet families were obtained implied that the response to mammatoxin is independent of organogenic competency.

However, in 10% of the ortet-ramet-extract combinations, significant differences in lesion diameter among ramets, and between some of the ramets and the ortet were obtained. The origin of intraclonal variation was unexplored, but experimental, physiological, and genetic variation or combinations thereof could be the underlying cause.

Despite the observation of intraclonal variation, when propagules regenerated from toxin-resistant cultures were compared to their respective donor plants in the leaf puncture bioassay, all trees in all clones exhibited an identical, resistant response. This was also the case in clones regenerated on toxin-containing medium when the toxin response of the donor plant was unknown. The infallibility of this observation suggests the ability to respond morphogenically on medium containing mammatoxin is an absolute requirement for resistance.

In this regard, the tissue culture system does not induce variation, but merely allows for isolation and subsequent propagation of the natural resistance already present. This technique may allow for clonal propagation of mammatoxin-resistant aspen, or, provide an <u>in vitro</u> bioassay to screen or evaluate seedlots of quaking aspen.

As noted in the introduction, mammatoxin has only been putatively identified as a determinant in the disease. It is only by analogy to other diseases employing host-selective toxin determinants that it can be stated that resistance is correlated (even in closely related nonhosts) with the extent of necrosis produced in a bioassay. However, the outstanding success in *in vitro* phytotoxin selection imparting disease resistance for toxin determinant diseases of agronomic crops suggests that screening with mammatoxin is a viable approach if a deterministic role is confirmed.

Any confirmation of the ability of mammatoxin resistance to impart resistance to infection (or subsequent virulence) of hypoxylon canker will probably have to occur through observation of infection by natural means. In this regard, French[71] found that response in the bioassay was not correlated with infection by artificial inoculation, even on a clone that appeared to have a response similar to cottonwood or the toxin-resistant clones isolated in this work. However, the previously mentioned difficulties with artificial inoculation should be recalled, as well as the study by Manion *et al.*[75] in which the mammatoxin leaf puncture bioassay correlated to natural infection, whereas results obtained with artificial inoculation were not. Unfortunately, as with many other traits in forest species, years are required before any correlations of toxin response with disease incidence or severity can be made. In the case of hypoxylon canker, the disease only begins to develop when the trees reach pole size (10 years).

Potential problems in the correlation of the bioassay and natural canker incidence of hypoxylon in progeny groups are illustrated by preliminary attempts undertaken here (Table 19). Natural canker incidence in Table 19 was based on 20-year observations averaged over a wide variety of sites near Clintonville,

Glidden, Greenville, Sugar Camp, and Wausaukee, WI. In some progeny groups, the rate of new infections had declined to near zero by 20 years, and in these cases the estimated percentage of infection was asymptotically extrapolated.

Table 19. Comparison of natural incidence of hypoxylon canker to the mammatoxin response for various full-sib progeny groups.

Parentage ♀	Parentage ♂	Project 3537 Rating	Hypoxylon Canker Incidence, %[a]	Resistance, %[b]
T-50-60	T-44-60	M	30	16[d]
XT-22-56,S2	T-44-60	M	--	28
T-20-56	T-46-60	M	--	24
T-1-58	T-6-61	M	25[c]	8
T-5-61	T-44-60	MH	30	12[d]
T-53-60	T-44-60	MH	--	32
T-50-60	T-20-60	H	55	4[d]
T-5-61	T-20-60	H	55	12[d]
T-24-60	T-13-58	H	85[c]	12
T-1-58	T-20-60	H	55[c]	44

[a]Minimum 20-year evaluation.
[b]Percentage of individuals exhibiting response identical to *P. deltoides* as determined by the leaf puncture bioassay.
[c]Percentage infection derived from asymptotic extrapolation.
[d]Data kindly provided by IPC Project 3501-3.

Linear regression on the percentage of toxin resistant individuals and natural infection levels showed no significant correlation ($r = 0.11$). In retrospect, this may not be surprising when it is considered that the mammatoxin bioassay is independent of space and time. Presumably, "space" dependence in terms of site, stand density, and other biotic and nonbiotic factors are eliminated by growing trees in the same medium in a greenhouse. The time dependence

might best be considered as the rhetorical question, "If given enough time, would the infection level rise to a value comparable to the percentage of resistant individuals"? In some cases, it is not inconceivable that the amount of time required would be so long that the trees might be in a state of decline, and other factors could contribute to infection. In any event, for pulpwood, knowledge of infection levels at 20-25 years has more practical value than resistance at 60-80 years.

An additional problem with the leaf puncture bioassay, as it was practiced here, is in the general inability to distinguish between necrotic responses of varying degrees of severity. It may be recalled that other studies have employed this bioassay to detect differences in necrotic responses, and it has been positively correlated to the incidence of natural infection. "Hypersensitivity," as measured by early branch death, was also positively correlated to canker elongation rate, suggesting that the toxin may be involved in virulence. Examination of IPC Project 3250 data on the time dependence of infection incidence also indicates differences in infection rate, suggesting the simple rating system employed reflects real differences for "medium" vs. "high" ratings. However, examination of Fig. 10 reveals little variation in the percentage of individuals with lesion diameters on the order of 3-6 mm. Thus, real differences in virulence may exist and be detectable by the bioassay, but not in this work. The sampling of one less than fully expanded leaf was part of the experimental procedure, as it was considered the most sensitive test of resistance. However, at the same time, the tendency for these leaves to be more sensitive to the toxin may have introduced substantial variation into the mean lesion diameter reported for each extract. In retrospect, crosses with a "low" rating should have been examined to potentially increase the likelihood that differences in the degree of

necrosis might have been detected. One consistent feature exhibited in all the seedlots was that all were susceptible to hypoxylon canker to a significant degree. This might be reflected in the bioassay by the observation that the percentage of toxin susceptible individuals exceeded the percentage of resistant individuals in every cross examined.

A possible working hypothesis that arises from this observation may account for the lack of correlation between the bioassay and the extent of natural infection. If toxin resistance imparts disease resistance, then it is not surprising that in all the crosses examined, substantial hypoxylon levels are observed because the level of infection at 20 years has not reached a level commensurate with the percentage of resistant individuals.

In this regard, it is interesting to note that for the three crosses in Table 19 where an asymptotic infection level was reported, in two of these the asymptote is associated with the percentage of resistant individuals (T-1-58 x T-20-60, XT-20-84; T-24-60 x T-13-58, XT-2-84).

Based on the above considerations, the bioassay response of "low" crosses can be predicted. The two possible situations are:

1. Low crosses contain $\geq$ 50% resistant individuals (lesion diameter $<$ 1 mm), but the remaining portion of the population responds to the toxin to the same extent as susceptible individuals in the crosses examined. This would result in a time-dependent plot of infection level that would rise at 1-2 percent per year (just as in medium and high crosses) but the infection rate would begin to decline sooner than 20 years.

2. Low crosses contain > 50% resistant individuals, but the remaining portion of the population is only slightly sensitive to the toxin. This implies reduced virulence such that infection would proceed at a rate slower than 1%. The infection rate would be small at 20 years, but would show no signs of decreasing.

Preliminary examinations of natural infection rates for low crosses reveal that the second situation appears to be the case.

The in vivo observation that callus formation is a resistance response to natural infection and in vitro bud differentiation is a criterion for mammatoxin resistance suggest similarities between these two processes. Although outward appearance does little to suggest similarities between developing shoot primordia and callus on wounded trees, in both cases early dedifferentiational events are likely to be the same. In trees, these events lead to the formation of rows or sheets of wound cambium that later give rise to rudimentary xylem elements. In seedling explants, cell division occurs throughout the entire structure, but gives rise to nodular regions of intense divisional activity. However, under the particular hormonal regime imposed, redifferentiation results in shoot primordia. Both processes have early events characterized by rapid cell division, leading to a decrease in cell size and an appearance characteristic of a meristematic state. Subsequent redifferentiation from this state in both cases becomes a function of the environmental (particularly hormonal) conditions.

The demonstration that the leaf puncture bioassay and the in vitro bioassay are both capable of identifying resistant individuals reiterates the possibility that the toxin acts on processes fundamental to all cells. However, the criteria

that form the basis of evaluating the response are quite different. Indeed, leaf necrosis has been considered a poor criterion for evaluation. Because necrosis is indicative of death without regard to causality, it is often regarded as a secondary response, perhaps unrelated to primary deterministic events that occur. In the case of mammatoxin, the 48-hour requirement for assay resolution also suggests that necrosis is a secondary event. When seedlings are explanted onto toxin-containing shoot proliferation medium, no extensive necrosis appears, but susceptible explants do lose their green color, appear sickly, and undergo very little morphogenesis. However, suggestions that the cultures fail to develop because the toxin is directly inhibiting callus formation, is, like leaf necrosis, making assumptions based on secondary effects. As in necrosis, callus formation (and bud differentiation) is a complex series of events, some of which might be altered in a way not related to the observed response. In the case of the in vitro bioassay it is not difficult to see why morphogenesis is limited because the cultures appear to be dying. It therefore seems likely that both bioassays represent secondary effects that, when absent, can only be construed that no primary, deterministic event occurred.

Whatever the relationship between toxin and disease resistance is, in an abstract sense this represents the first demonstration in a woody species that plants can be regenerated from stress-tolerant cultures and retain the stress tolerant response. However, greater significance probably lies in the observation that the desired response was already present to a significant degree in the population. Thus, for disease resistance, in vitro manipulations in attempts to induce, mutate, or transform cell cultures of woody species to obtain disease resistance may be largely a misdirection of effort. A better expenditure of effort would be aimed at isolation of natural resistance and more importantly,

its subsequent mass propagation. In this study, shoot cultures could be established from toxin-resistant clones that seemed to have the potential for providing a continuous harvest of rootable shoots. However, at the current stage of development, it is unlikely that mass clonal propagation by this method would be cost-effective even with the added value associated with the potential doubling of volume growth/acre. As a result, the only current application this method seems to have is to screen promising progeny groups for toxin resistance, followed by future evaluation not only for hypoxylon resistance, but form and growth rate as well. Individuals containing coincidence of these traits would then be integrated into an existing breeding program with the hope that resistance could be passed on to the F2 generation.

Until the capacity for and problems associated with mass production are resolved, it seems likely that application of in vitro technology in woody species will be similar to the potential applications mentioned here. In other words, tissue culture may serve as a valuable tool for tree improvement, but it may not become an end unto itself and usher-in a new era of "modern forestry."

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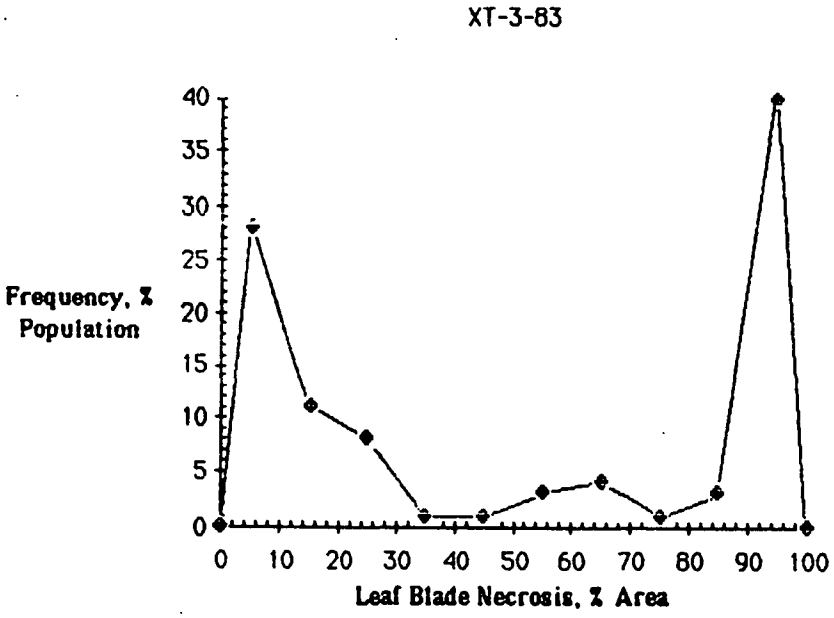
APPENDIX



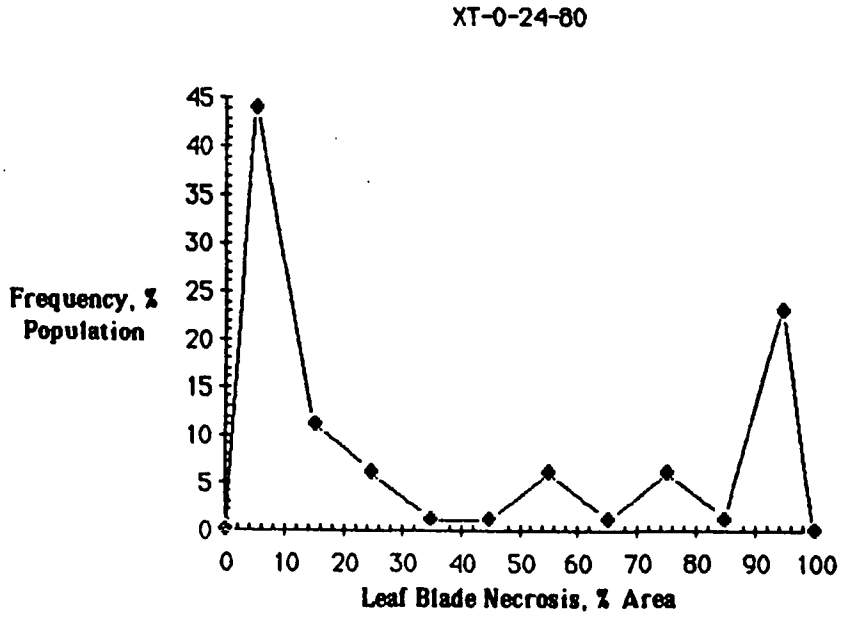


Figure 20. Whole leaf bioassay of seedlings (N = 65).

Table 20. Media formulations employed in micropropagation of aspen from seedling explants.

Component	mg/L Bud Initiation (MS)[a]	Shoot Elongation (1/2 MS)	Root Formation (1/3 MS)
NH_4NO_3	1650	825	550
KNO_3	1900	950	633
KH_2PO_4	170	85	57
$CaCl_2$	440	220	147
$MgSO_4 \cdot 7H_2O$	370	185	123
H_3BO_3	6.2	3.1	2.1
KI	0.83	0.42	0.28
$MnSO_4 \cdot H_2O$	16.9	8.5	5.6
$ZnSO_4 \cdot 7H_2O$	8.6	4.3	2.9
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.13	0.083
$CuSO_4 \cdot 5H_2O$	0.025	0.013	0.0083
$FeSO_4 \cdot 7H_2O$	27.8	13.9	9.3
Na_2 EDTA	37.3	18.7	12.4
myo-Inositol	100	50	33
Nicotinic acid	0.5	0.25	0.17
Pyridoxine•NCl	0.1	0.05	0.033

See end of table for footnote.

Table 20 (Continued). Media formulations employed in micropropagation of aspen from seedling explants.

Component	mg/L Bud Initiation (MS)[a]	Shoot Elongation (1/2 MS)	Root Formation (1/3 MS)
Thiamine•HCl	0.1	0.05	0.033
Sucrose	30,000	15,000	10,000
Napthalene acetic acid (NAA)	0.1	—	—
N^6-Benzyl-adenine (BA)	1.0	0.3	—
Indole butyric acid (IBA)	—	—	0.1
Agar	8,000	8,000	6,500
pH	5.8	5.8	6.7

[a]Murashige and Skoog, 1962.

Table 21. Modified Fries' medium employed for liquid culture of Hypoxylon mammatum.

Component	mg/L
$MgSO_4$	493
$CaCl_2$	132
KH_2PO_4 (pH = 6)	1,361
H_3BO_3	0.68
$CoCl_2$	0.16
$CuSO_4 \cdot 5H_2O$	0.08
$Na_2MoO_4 \cdot 2H_2O$	0.09
$MnSO_4 \cdot H_2O$	0.30
$ZnSO_4 \cdot 7H_2O$	0.10
$FeSO_4 \cdot 7H_2O$	1.25
Na_2 EDTA	1.68
Biotin	0.01
Thiamine·HCl	0.1
Potassium glutamate	4,630
D-glucose	5,000

Table 22. Mammatoxin response of full-sib aspen crosses (leaf puncture bioassay, 18 weeks, XT-2-84)[w].

Tree No.	H^+ EtOAc Mean Lesion Diameter, mm	Tree No.	H^+ n-BuOH Mean Lesion Diameter, mm
3	5.2 a	12	4.6 a
25	5.1 a	10	4.6 a
14	4.8 a	3	4.3 a b
16	4.8 a	21	4.2 a b c
13	4.7 a b	17	4.1 a b c d
21	4.6 a b	16	4.0 a b c d
17	4.5 a b	25	3.8 a b c d e
5	4.4 a b	13	3.9 a b c d e
10	4.4 a b	14	3.9 a b c d e
12	4.4 a b	5	3.2 a b c d e f
1	4.1 a b c	1	2.9 a b c d e f
23	3.8 a b c d	22	2.4 a b c d e f
11	3.7 a b c d	24	2.2 a b c d e f
22	3.7 a b c d	2	1.9 b c d e f
24	3.2 a b c d e	11	1.8 b c d e f
2	3.0 a b c d e f	8	1.7 b c d e f
9	2.6 b c d e f	20	1.7 c d e f
8	2.5 b c d e f	23	1.6 d e f
15	2.0 c d e f	9	1.3 e f
19	2.0 c d e f	4	1.2 f
4	1.7 d e f	19	1.1 f
7	1.3 e f	7	1.1 f
20	1.2 e f	6	0.89 f
18	1.0 e f	15	0.89 f
6	0.89 f	18	0.83 f

[w]Within a column, means followed by common letters are not significantly different by one-way ANOVA with Duncan's Multiple Range Test ($p = 0.05$).

Table 22 (Continued). Mammatoxin response of full-sib aspen crosses (leaf puncture bioassay, 18 weeks, XT-13-84).

Tree No.	H^+ EtOAc Mean Lesion Diameter, mm	Tree No.	H^+ n-BuOH Mean Lesion Diameter, mm
23	5.8 a	11	5.5 a
7	5.8 a	19	5.3 a
25	5.8 a	23	5.1 a b
8	5.6 a	7	4.6 a b c
11	5.6 a	9	4.6 a b c
19	5.3 a	22	4.6 a b c
4	5.2 a	4	4.2 a b c d
21	5.2 a	12	4.2 a b c d
24	4.5 a b	8	4.1 a b c d
10	4.3 a b	20	3.8 a b c d
3	4.2 a b	25	3.8 a b c d
9	4.2 a b	17	3.7 a b c d
17	4.1 a b	24	3.6 a b c d
6	3.9 a b	10	3.3 a b c d e
12	3.9 a b	13	3.3 a b c d e
5	3.7 a b c	3	3.2 a b c d e
16	3.6 a b c	16	2.7 b c d e
13	3.6 a b c	6	2.7 b c d e
20	3.5 a b c	21	2.6 c d e
2	2.6 b c d	5	2.2 c d e
22	2.5 b c d	15	2.1 c d e
15	2.4 b c d	2	1.9 d e
18	2.1 b c d	1	1.1 e
1	1.3 c d	18	0.94 e
14	0.83 d	14	0.89 e

Table 22 (Continued). Mammatoxin response of full-sib aspen crosses (leaf puncture bioassay, 18 weeks, XT-6-80).

Tree No.	H^+ EtOAc Mean Lesion Diameter, mm	Tree No.	H^+ n-BuOH Mean Lesion Diameter, mm
3	9.7 a	3	7.9 a
22	7.7 a b	2	7.3 a b
2	7.6 a b	1	6.7 a b c
1	7.3 b	22	6.3 a b c
11	7.3 b	11	5.8 a b c
17	7.0 b	15	5.8 a b c
18	6.8 b	9	5.8 a b c
15	6.4 b c	17	5.4 a b c d
9	6.3 b c d	18	5.3 a b c d
10	6.2 b c d	10	4.9 b c d e
19	5.6 b c d e	24	4.7 b c d e f
24	5.6 b c d e	14	4.4 c d e f
7	5.6 b c d e	25	4.4 c d e f
25	5.6 b c d e	19	4.4 c d e f
14	5.3 b c d e	4	2.8 d e f g
4	4.1 c d e f	7	2.8 d e f g
20	3.9 d e f g	20	2.8 d e f g
21	3.2 e f g h	13	2.4 e f g
8	2.6 f g h i	21	2.0 f g
5	2.1 f g h i	23	1.6 g
13	1.7 g h i	16	1.4 g
16	1.7 g h i	5	1.4 g
23	1.1 h i	6	1.2 g
6	0.89 h i	8	1.1 g
12	0.73 i	12	0.89 g

Table 22 (Continued). Mammatoxin response of full-sib aspen crosses (leaf puncture bioassay, 18 weeks, XT-4-82).

Tree No.	H^+ EtOAc Mean Lesion Diameter, mm	Tree No.	H^+ n-BuOH Mean Lesion Diameter, mm
12	5.6 a	12	4.9 a
4	5.5 a	19	4.5 a b
19	5.4 a	21	4.4 a b
7	5.0 a b	8	4.3 a b
6	4.9 a b	13	4.2 a b
13	4.8 a b	14	4.1 a b c
18	4.8 a b	6	2.9 a b c d
8	4.7 a b c	4	2.8 a b c d
21	4.6 a b c	17	2.7 a b c d
11	4.4 a b c	7	2.5 b c d
17	4.3 a b c	11	2.5 b c d
2	3.9 a b c	5	2.4 b c d
9	3.6 a b c	9	2.4 b c d
14	3.6 a b c	2	2.3 b c d
5	3.2 b c	18	1.9 c d
22	3.2 b c	22	1.9 c d
1	2.6 c d	1	1.2 d
20	1.1 d	3	1.1 d
24	1.1 d	23	1.1 d
23	0.94 d	10	1.0 d
10	0.89 d	24	0.94 d
3	0.83 d	15	0.89 d
15	0.78 d	20	0.83 d
25	0.72 d	16	0.78 d
16	0.67 d	25	0.78 d